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Award Number: DAMD17-94-J-4495

TITLE: New Therapeutic Approaches and Prognostic Assays for Breast Cancer: Radiolabeled Ligands and Antibodies and Quantitative PCR

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Washington, DC 20059

REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Annual (30 Sep 98 – 29 Sep 99)

4. TITLE AND SUBTITLE
New Therapeutic Approaches and Prognostic Assays for Breast Cancer: Radiolabeled Ligands and Antibodies and Quantitative PCR

5. FUNDING NUMBERS
DAMD17-94-J-4495

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)*** The presence of estrogen receptor (ER) alpha in breast tumors is used as a marker to predict prognosis and treat patients with anti-estrogen therapies. The rate of response to anti-estrogen therapies is about 50%. Recent studies have shown that tumor cells express several forms of ER suggesting that the composition of various isoforms in the tumor cells may correlate with response/resistance to anti-estrogen therapies. Currently, the tumor tissues are diagnosed for the presence of ER alpha by immunohistochemistry The problem is that this method is not sensitive to detect small amounts of ER protein and cannot distinguish and quantify various ER isoforms. For these reasons, there is an urgent need to develop a highly sensitive assay that can rapidly quantify various ER isoforms. We proposed to develop PCR based molecular assays that can quantify ER mRNAs. We used reverse transcription template competition approach to devise an assay and showed that it could be applied to quantify the major tumor ER mRNA, the alpha isoform, in the tumor tissues. Since only a few of the alternatively spliced ER mRNAs could be identified by co-amplification with the wild type ER, we developed a 'Splice Targeted Primer Approach' to specifically amplify the splice variants with out amplifying the wild type. Using this novel approach, we identified twenty different alternatively spliced ER mRNAs in breast cancer cell lines and tumors many of which were not known previously. We have also developed methodologies that can quantify the alternatively spliced ER alpha mRNAs as separate gene populations using the splice targeted primers and template competition PCR. These methods will be highly valuable in establishing a correlation between the expression of various ERs in a tumor with response/resistance to anti-estrogen therapies.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Hormone Receptors, Quantitative PCR, Radiolabeled PRL, Humans, Anatomical Samples	84
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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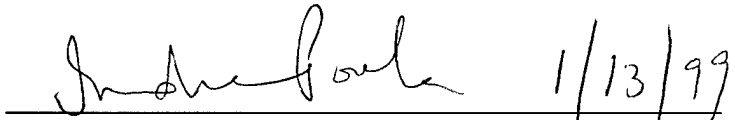

PI - Signature Date 1/13/99

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GRANT REPORT
Indra Poola, Ph.D

1. INTRODUCTION

Breast cancer is a tumor of the mammary gland epithelium affecting about 176,000 women in the western hemisphere and killing about 50,000 people every year in the United States. A substantial body of epidemiological, experimental, and clinical evidence indicates that exposure to the natural hormones, estrogen, progesterone and prolactin, which are important for the normal development and function of the breast tissue, play a major role in the growth of breast cancer cells and may even contribute to the development of breast cancer.

A. Prognostic factors in breast cancer. Once diagnosed with breast cancer, therapeutic approaches, survival rate and disease recurrence depend upon the expression of the prognostic factors, hormone receptors, growth factor receptor superfamily and proteins involved in invasive metastatic phenotype in the tumor tissues.

B. Hormone receptors in the prognosis and therapy of breast cancers. The most important among prognostic factors are the hormone receptor, estrogen-, and progesterone receptors (ER and PgR). Estrogen receptor. The presence of ER in tumors indicates a good prognosis and the patients respond to anti-estrogen therapies. The expression of various molecular forms of ER was studied in several breast cancer cell lines and tissues. Analysis of the estrogen receptor mRNA has shown that it undergoes alternate / inaccurate splicing in the tumor tissues giving rise to several aberrant (variant) forms of the receptor molecules. These studies raise the possibilities of various species of ER which have exon deletions/truncations in the 1) estrogen binding region (exons 4-8), 2) DNA binding region (exons 2-3) and 3) other portions of the receptor molecule.

C. Current methods of estrogen receptor evaluation and their disadvantages.. The presence of estrogen receptor is the basis for anti-estrogen therapies to treat breast cancer and its presence also indicates good prognosis, longer survival and low recurrence rates. Because of these therapeutic and prognostic reasons, estrogen receptor is detected and quantitated in tumors. Currently its detection and quantification are done using immunohistochemistry procedures in clinical laboratories.While these provide information on the presence of ER, they are 1) very cumbersome, 2) time consuming, 3) not highly sensitive, 4) require a large sample 5) **do not yield quantitative information**, 6) not suitable to evaluate in fine needle aspirations 7) very expensive to the patient and 8) **Most importantly, immunohistochemical assays cannot distinguish between the wild type and variant forms of ER, therefore, cannot predict hormone therapy responders precisely. Because of the therapeutic considerations, there is an urgent need to rapidly quantitate and get a profile of wt and vt of ER to predict hormone therapy responders and disease prognosis.**. Furthermore, due to increased awareness and improved methods of detection, smaller and smaller tumors are detected in recent times which restricts the amount of tumor tissue available for the immunohistochemisty. This led to a proposal that molecular biological methods may be preferred since they require very small samples. In addition, several prognostic factors could be detected at the same time by these procedures.

The essence of our proposal is to develop highly sensitive cost effective quantitative PCR assays to measure a multitude of hormone receptors and other diagnostic and prognostic factors in breast cancers.

D. Development of new prognostic assays for estrogen receptor. In our grant, we proposed to develop highly sensitive, rapid, cost-effective PCR based methods to quantitate ER and other prognostic factors in a small amount of clinical samples. The developed assays could be used virtually in every clinical lab to diagnose and evaluate prognosis of breast cancers. The significance of this assay is that patients could then be identified who are most likely to respond to hormone therapy and that the overall prognosis may be assessed.

2. SPECIFIC AIMS.

The specific aims of our proposal are to develop highly sensitive PCR methods for rapid, specific, diagnosis of breast cancers on the basis of their estrogen- and progesterone receptor expression.

3. BODY OF THE REPORT (STUDIES AND RESULTS).

We have developed the state-of-the art molecular methods for the detection and quantification of estrogen receptor in breast tumors. In particular we have developed molecular procedures to

1) detect and quantify wild type estrogen receptor alpha mRNA, 2) analyzed the alterations in the estrogen receptor mRNA in the breast tumors of Caucasian and African American women 3) devised novel "Splice Targeted Primer Approach" for detecting the estrogen receptor alpha mRNAs which have deletions in one or more exons 4) Identified twenty alternatively spliced ER alpha mRNAs which have deletions in various combinations of exons in breast cancer cell lines and tumors and 5) developing methods for the quantification of estrogen receptor exon-deletion variant mRNAs in breast cancer cell lines and tumors. These accomplishments are described briefly here.

A. Development of molecular methods to quantitate estrogen receptor mRNA copy numbers. We have devised a method to quantify the mRNA copy numbers of estrogen receptor by reverse transcription polymerase chain reaction (RT PCR) template competition method. We were the first to develop the quantitative molecular method for the quantification of the receptor mRNA in tumor samples. The procedure is described in detail in the accompanying publication.

Our work on the quantitation of estrogen receptor copy numbers is published in Analytical Biochemistry. A copy of the paper is attached.

B. Studying the estrogen receptor profiles in the breast tumors of Caucasian and African American women. After developing the quantification methods for the ER wild type mRNA, we wanted to apply these methods for the quantification of ER in tumor samples. Therefore, we started collecting the tumor samples from Howard university hospital where a large number of breast tumor biopsies are performed. Since the majority of biopsies performed at Howard University hospital are on African American women, it gave us an opportunity to look at any alterations in the ER in these samples. Several reports indicate that the incidence of breast cancer in young African American women is higher and lower in older women compared to Caucasian women. However, the mortality rate with breast cancer is three times as high in African American women compared to women in other populations. The high mortality rate does not appear to be due to differences in socioeconomic status, stage of diagnosis, age, known risk factors or treatment. It has been hypothesized that breast tumors across racial and ethnic groups could have different biologic characters which may account for survival disparities. However,

such factors are not identified thus far. There also appears to be differences in tumor biology. The breast tumors from African American women in general are poorly differentiated with lower frequency of the steroid hormone receptors, the estrogen- and progesterone receptor, which are generally associated with well or moderately differentiated tumors and their presence indicate good prognosis. It is not known whether this aggressive tumor phenotype contribute to high mortality with breast cancer. All these studies indicate that the mortality rate is related to biological factors. Preventive and therapeutic strategies can be devised if those factors are identified in the tumors of African American women. Retrospective studies conducted on tumor tissues could be highly valuable in identifying those factors.

To understand the molecular factors which may be responsible for the poorly differentiated aggressive tumors in African American women, we studied the most important prognostic factor, the estrogen receptor in the tumor tissues. In particular, we investigated the alteration in the expression of estrogen receptor gene in the freshly collected breast tumors. Briefly, we studied the estrogen receptor mRNA in 25 immunohistochemically estrogen receptor positive and 5 immunohistochemically negative tumors from African American women and 20 ER-positive tumors from Caucasian patient tumors (obtained from Dr. Suzanne Fuqua at San Antonio, TX). We studied the receptor transcripts by reverse transcription polymerase chain reaction (RT PCR) using a number of primer pairs. Our results indicated that only a third of the tumors have full length receptor transcripts and the majority of tumors had at least three types of modifications. They are 1) truncations in exon 8, which encodes for part of the hormone binding region, 2) base insertions and 3) absence of naturally occurring exon-7 deletion variant. The significance of these finding and their role in the aggressive tumor types are not clear at this point.

This work has been accepted for publication in the Journal of Cancer Research and Clinical Oncology. Acceptance letter and a copy of the manuscript are enclosed.

C. A novel 'Splice Targeted Primer Design' for the targeted amplification of alternatively spliced ER mRNAs. Estrogen receptor occurs both as unmodified (wild type) and several spliced variants (isoforms) which have the deletions in one or more exons. Several studies have shown that the estrogen receptor isoforms play important functional roles in the estrogen induced signal transduction processes under normal physiological conditions. Recent reports also indicate that the isoforms of estrogen receptor pattern and levels are altered in breast tumors and their content influence the response to anti-estrogen therapies. Because of the importance of isoforms, it is important to develop methods which can precisely detect and quantify their amounts. The currently available methods can only detect the most abundant forms and cannot detect the mRNAs of the low abundance. Therefore, we have developed new strategies to detect even the least abundance isoform by PCR using targeted primers. In particular we designed, tested and identified targeted primers for exon-7 deletion variant, exon-5 deletion variant, exon 2-deletion variant and exons 2-3 deletion variants.

These results are published in Analytical Biochemistry. A copy of the reprint is enclosed.

D. Identification of twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using "Splice Targeted primers". After developing the new primer design strategies for specific amplification of the mRNAs of isoforms, we used those targeted primers to analyze the variant patterns in breast cancer cell lines and tumor samples. We

employed seven breast cancer cell lines, MCF-7, T47D, ZR-75,LCC1, LCC2, LCC9 and MDA-MB-435 and three tumors to test the applicability of the targeted primers. Our results showed that the targeted primers not only amplified the single deletion transcripts, but also multiple deletion isoforms. These transcripts were not detected and characterized until now because of the unavailability of the sensitive methodologies until now. The different cell lines did not differ in the single deletion transcripts. However, they differed in the presence and content of multiple deletion transcripts. Interestingly, we observed only the multiple deletion variant transcripts in the tumor samples. The twenty spliced mRNAs identified were ***2Δ; 2Δ & 7Δ; 2Δ, 5Δ & 7Δ; 2Δ & 4-5Δ; 2Δ & 4-6Δ; 3Δ; 3Δ & 7Δ; 2-3Δ; 2-3Δ & 7Δ; 2-3Δ, 5Δ & 7Δ; 4Δ; 4Δ & 7Δ; 5Δ; 5Δ & 2Δ; 5Δ & 2-3Δ; 6Δ; 7Δ; 7Δ & 3-4Δ; and 7Δ & 3-5Δ*** (*Δ*, deletion). Our results also suggested that the alternative splicing takes place in a sequential manner instead of at random. Deletion of an exon in the transcript, the second exon to be deleted appears to be exon 7, which is followed by exon 5. Because of this trend, the most frequently deleted exon is the exon 7 and the second most frequently deleted exon is exon 5. The estrogen dependence appear to be associated with the presence of multiple deletion (exon 3-7 and exon 4-7).

The above work has been accepted for publication in the Journal of Steroid Biochemistry and Molecular Biology. A copy of the manuscript is enclosed.

E. Quantification of estrogen receptor isoform mRNAs. After establishing the applicability of variant targeted primers for specific amplification of variant transcripts, we decided to apply them in quantification of their transcripts by template competitive PCR similar to the quantification of wild type estrogen receptor transcripts.

A manuscript has been submitted and a copy enclosed.

4. KEY RESEARCH ACCOMPLISHMENTS.

- **Identified the alterations in the estrogen receptor mRNAs in the breast tumors of Caucasian and African American women**
- **Designed methodologies to detect and quantify ER mRNAs from a small amount of tumor tissue.**
- **Devised methods to specifically amplify the alternatively spliced estrogen receptor transcripts**
- **Identified twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors**
- **Developed methods to quantify alternatively spliced alpha mRNAs as separate gene populations.**

5. REPORTABLE OUTCOMES.

A. Publications and presentations:

1. I. Poola, D.M. Williams, S. Koduri, J. Ramprakash, R. E. Taylor and W.D. Hankins (1998) Quantitation of estrogen receptor mRNA copy numbers in breast cancer

2. D. M.Williams, S. Koduri, Z. Li, W. D Hankins, and I. Poola (1999) Primer design strategies for the amplification of alternatively spliced estrogen receptors. Analytical Biochemistry, 271, 194-197.

3. I. Poola, S. Koduri, S. Chatra and R. Clarke (2000) .Identification of twenty different alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using 'splice targeted primer approach'. J. Steroid Biochem. Mol. Biol. (In Press) .

4. S. Koduri, S. A. W Fuqua, and I. Poola (2000) Alterations in the estrogen receptor mRNA in the breast tumors of Caucasian and African American women. J. Cancer Res. and Clinical Oncol. (In press).

5. S. Koduri and I. Poola (2000) Quantification of alternatively spliced estrogen receptor mRNAs as separate gene populations (Submitted)

6. Microsatellite instability and loss of heterozygosity in chromosome 13q12-13 in ductal carcinoma in situ of the breast. Y. Huang, I. Russo, I. Poola, E. B. Chung,, and J. Russo (1998) Proceedings of 89th Annul meeting, American Association for Cancer Res. A3651

B. Patients and Licences

None

C. Degrees awarded

None

D. Development of cell lines and tissues

None

E. Informatics such as databases

None

F. Funding applied for based on work supported by this grant.

1. Changes in the alternatively spliced estrogen receptor alpha mRNAs in the breast tumors compared to normal breast tissues.

Applied to Susan G. Komen breast cancer foundation and awarded $141,000.

2. Clinically applicable molecular assay to profile estrogen receptors in breast tumors Applied to National Cancer Institute on November 22, 1999.

G. Employment or research opportunity applied for

None

6. CONCLUSIONS. A highly sensitive molecular biological approaches have been developed to detect and quantitate the exact copy numbers of wild type and various single and multiple exon deletion forms of estrogen receptor mRNA in breast tumors. Once converted into a clinically feasible assay, it could become a highly valuable method to precisely predict the prognosis of the disease and identify patients who are most likely to respond to anti-hormone therapy.

7. APPENDICES.

Two reprints, two manuscripts which are in Press and one manuscript which has been submitted.

QUANTITATION OF ALTERNATIVELY SPLICED ESTROGEN RECEPTOR ALPHA mRNAS AS SEPARATE GENE POPULATIONS

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The estrogen receptor (ER) alpha mRNA undergoes alternate splicing, generating transcripts with deletions in exons 2-, 3-, 2-3, 4-, 5-, 6- and 7. The presence of ERα splice variants has been described both in normal and malignant tissues (1, 2). Several reports suggest that the alternatively spliced ERs are involved in estrogen-induced signal transduction processes, contribute to malignant transformation and hormone resistance (3-5). In order to precisely define their role in biology and medicine, new methods are needed which can, not only detect and distinguish them from the wild type, but also quantitate the proportion of alternatively spliced ERs in a mixed population. Conventionally, the ER exon deletion variant transcripts are detected and quantitated by co-amplification with the wild type sequences using RT-PCR approaches. However, there are a number of practical limitations to this approach. Firstly, the threshold of detection- the spliced transcripts expressed at low levels fall below the levels of detection. Secondly, it cannot detect and quantify the transcripts which have deletions in distant exons such as exon 2- and 7Δ, Thirdly, since the wild type transcripts are present in large excess to alternatively spliced molecules, a competitive amplification occurs amongst the wild type and all the alternatively spliced transcripts. Therefore, quantification of splice variants by co-amplification does not yield the actual levels. To circumvent the problems involved in the detection of splice variants, we have devised a novel splice targeted primer approach which can specifically amplify the alternatively spliced mRNAs as separate gene populations without co-amplifying the wild type. We have shown that the targeted primers which have a minimum of 3 of 4 bases at the extreme 3' end unique for the splice junction will specifically amplify the spliced junction without amplifying the flanking wild type exons and in order to design such a primer, the

overhang sequences can extend up to eight bases past the splice junction (6). Using the splice targeted primer approach, we have detected twenty different ER variant mRNAs that have deletions in various combination of exons in breast cancer cell lines and tumors (7). In the current study we describe a method to quantify the absolute copy numbers of ER splice variant mRNAs by RT PCR template competition approach in combination with splice targeted primers using exon 7Δ mRNA as a model.

The template competition method of mRNA quantitation requires the construction of a competitor plasmid which has an extra fragment of an irrelevant DNA sequence internal to the PCR primer location that generates a larger PCR product than the template of interest and distinguishable by size exclusion gel electrophoresis (8). For the quantification of ER 7Δ mRNA copy numbers, we constructed a competitor in three steps. First, a 1kb length exon 7Δ mRNA was specifically amplified by RT PCR from an ER positive breast cancer cell line, LCC1, using splice specific anti-sense primer, ER AX6/8, 5' CTCCATGCCTTTGTTA 8/6 CAGAA 3' (positions, exon 8, bp 1801 to 1786 and exon 6, bp 1601-1597), and a sense primer, ERS, 5' TGCCCTACTACCTGGAGAACG 3' (position, exon 1, bp 615-635). The 1 kb exon 7Δ cDNA was cloned into pCR®II-TOPO (Invitrogen) vector as described before (7) to generate pCR®II-TOPO-ER7Δ. The sequence and locations of the ER primers described here are based on ER cDNA sequence published by Green and others (9). Second, the pCR®II-TOPO-ER7Δ was digested with *EcoR*I to release the cloned ER 7Δ fragment from two *EcoR*I sites which flank the insert and subcloned into an unique *EcoR*I site in pSG5. Third, a 366 bp fragment of DNA from bacteriophage λ was amplified and inserted into an

unique *Hind*III site in exon 4 of ER 7Δ fragment as described previously (10). The resulting competitor was termed as pSG5-ER7Δ- λ.

Next, the pSG5-ER7Δ- λ was used to determine the absolute copy numbers of ER 7Δ mRNA in LCC1 cell line by template competition RT PCR approach. The PCRs were conducted with a constant amount of LCC1 cDNA, prepared from 250 ng of RNA and in the presence of increasing amounts of the competitor (10^5 to $10^{6.75}$ copies in quarter-log dilutions). The PCRs were performed in 25 μL as described previously (6-7, 10) and the products were electrophoresed in 4.5% acrylamide gels and visualized by ethidium bromide staining. A typical quantitation assay performed for ER 7Δ in LCC1 cells is shown in Figure 1. As seen in Figure 1, the amount of 1 kb ER 7Δ product (lower band) decreased in the presence of increasing amounts of the 1.366 kb competitor, pSG5-ER7Δ- λ product (upper band). To determine the exact copy numbers of ER 7Δ, first, the fluorescence intensity of the above products was quantitated densitometrically using Mustaq 1200-3E scanner and NIH image 1.60 program. Second, the scanning units were normalized to the size of the competitor. Third, the normalized scanning units were plotted against the number of copies of the competitor template in the PCR. A representative graph is shown in Figure 2. A cross-over point at which the competitor and the reverse-transcribed cDNA gave equal amounts of PCR products was 2.2 X 10^5 copies. Therefore, the number of copies of ER 7Δ in LCC1 cDNA prepared from 250 ng of total RNA was 2.2 X10^5 copies. These numbers were repeatable in several experimental trials.

We believe that the above described template competition RT PCR method of quantification in combination with splice specific primers could be applied to quantify exact mRNA copy numbers of all ER splice variants except ER 4Δ mRNA, since it lacks the unique *Hind*III site to construct a competitor. A competitor for ER 4Δ can be constructed by inserting λ sequence into an unique *Bgl*II site in exon 6. We believe that the quantitative data on ER variant mRNAs will be highly useful in determining the role of both naturally occurring and tumor expressed ER spliced variants in binding and response to various natural and synthetic hormones and understanding the hormone independence in breast cancer.

Acknowledgements. This work was supported by grants from the Susan G.Komen Breast Cancer Foundation and the Department of Defence (DAMD 17-94-J-4495) awarded to I. P. The technical assistance of Zang Li is acknowledge. Dr. R. Hakim is acknowledged for his help in the use of NIH Image 1.6 program.

Abbreviations. ER, Estrogen receptor; Exon Δ, Exon deletion.

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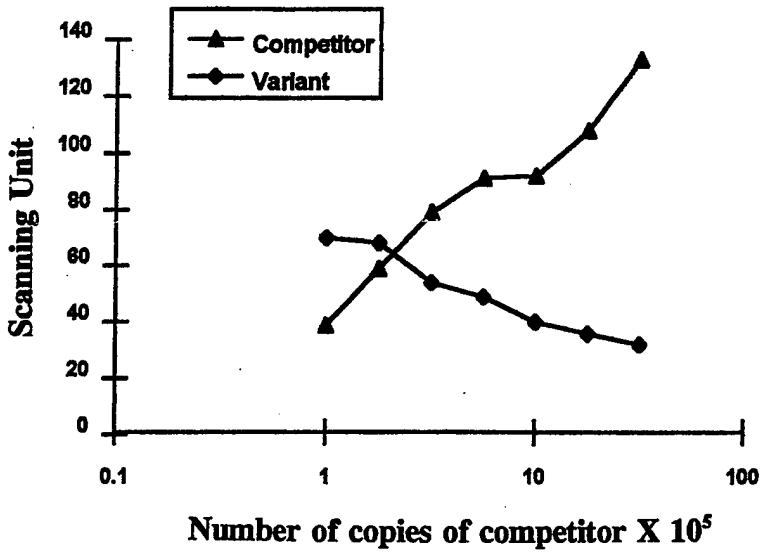
FIGURE LEGENDS

Figure 1. Quantification of ER exon 7Δ mRNA copy numbers by template competition RT PCR approach using splice targeted primer. Template competition assay was performed with constant amount of cDNA prepared by reverse transcription of 250 ng of LCC1 total RNA and in the presence of varying amounts of the competitor pSG5-ER7Δ- λ as described in the text. The amplified products were electrophoresed in 4.5% acrylamide gels and detected by ethidium bromide staining.

Figure 2. Absolute quantitation of ER 7Δ copy numbers in LCC1 cells. The intensity of the PCR products was measured by scanning the photographs of the ethidium bromide stained gels in a Mustaq 1200-3E scanner and quantitated using NIH Image 1.60 program. The scanning units were normalized to the size of the competitor and the normalized scanning units were plotted against the number of copies of the competitor, pSG5-ER7Δ- λ, in the PCR reaction. The cross-over point is shown at which the specific ER 7Δ product generated from LCC1 and the competitor DNA are equal. The number of copies of ER 7Δ copy numbers is equal to the number of copies of the competitor at the cross-over point (2.2×10^5).

Quantitation of ER exon 7Δ



Quantitation of ER exon 7Δ

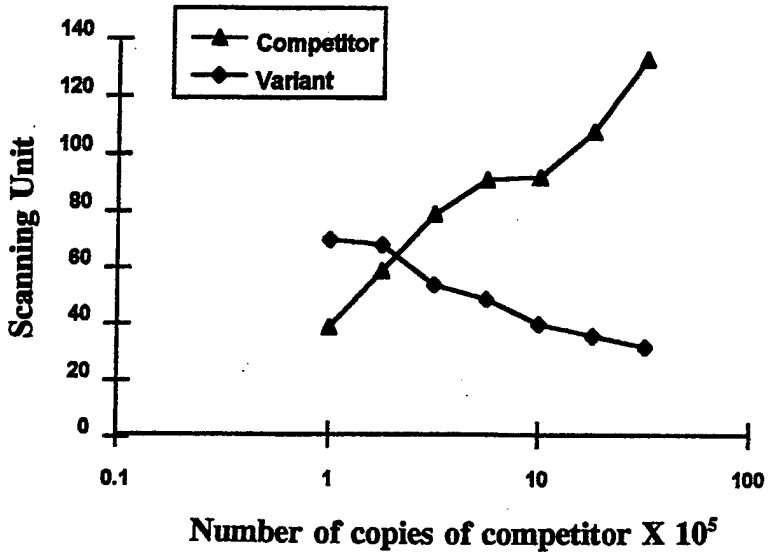


Figure 1

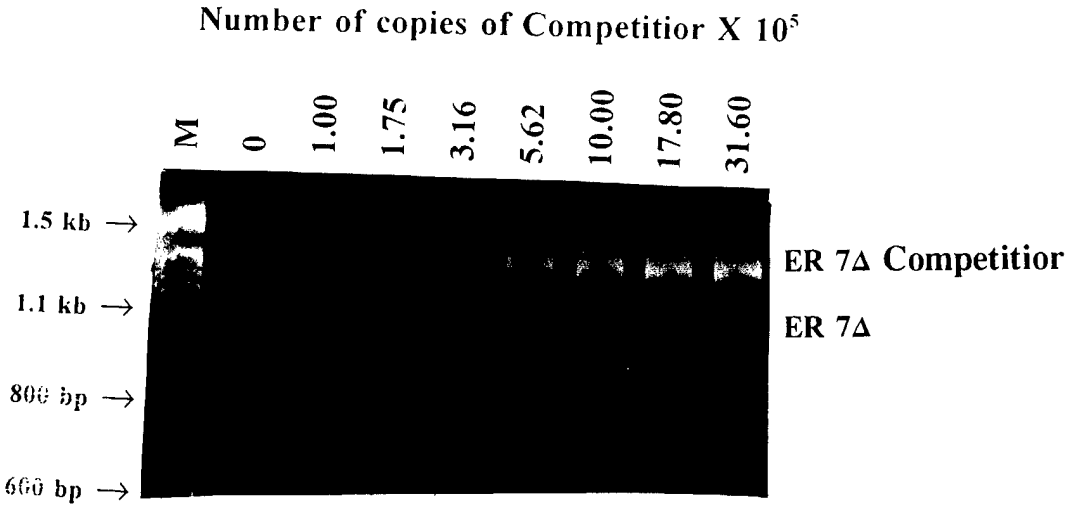
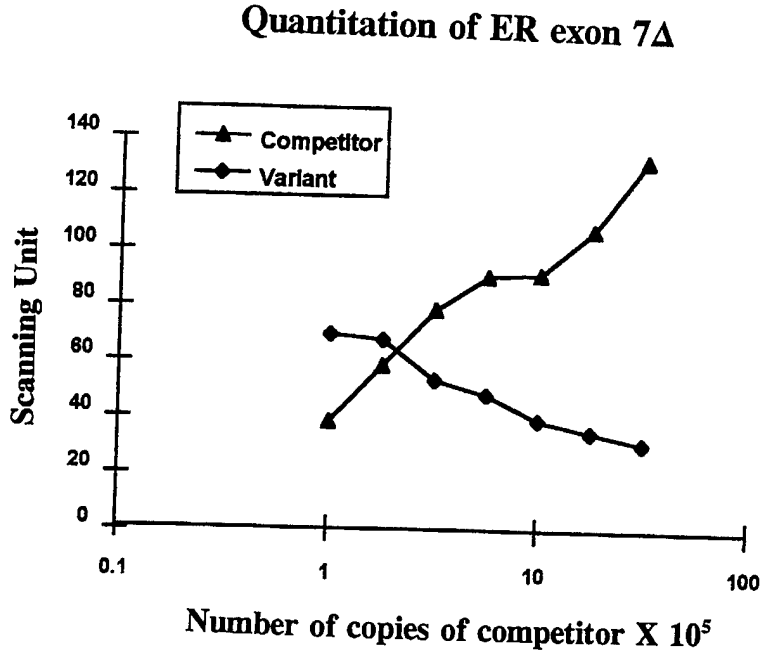


Figure 2



ANALYTICAL BIOCHEMISTRY 258, 209–215 (1998)
ARTICLE NO. AB982629

Quantitation of Estrogen Receptor mRNA Copy Numbers in Breast Cancer Cell Lines and Tumors

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Received November 20, 1997

Several clinical studies have suggested that the content of estrogen receptor (ER) in breast tumors influences the survival, tumor recurrence, and response to antiestrogen therapies. Therefore, the ability to precisely quantitate the ER content in tumor tissues will be of significant benefit to women with breast cancer. Although immunohistochemical and polymerase chain reaction (PCR) methods have been described for the detection and semiquantitation of ER, none of them precisely quantitate ER copy numbers in tumor samples. In the present report we describe a molecular approach to accurately quantitate ER mRNA copy numbers using a reverse-transcription PCR (RT-PCR) template competition method. A competitor template was devised by inserting unrelated nucleic acid sequences into an ER cDNA clone. A template competitive RT-PCR analysis was then performed to determine the number of copies of ER mRNA. As a standard of reference for the ER mRNA copy numbers from various samples, the mRNA copy numbers of a constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were also quantitated. The ER quantitations were performed in three positive cell lines, MCF-7, T47D, and ZR-75, and two positive tumor tissues by this approach. Our results described here show that among the cell lines studied, T47D expresses the highest copy numbers of ER. We also present here that ER as low as 10^3 copies per 10^5 copies of GAPDH can be detected and quantitated in tumor samples by the template competition method. In addition, the molecular approach can simultaneously detect, distinguish, and quantitate exon deletion variant copy numbers of ER. The results described in this report indicate that the ratios of exon 7 deletion variant to wild type in the tumor tissues are significantly higher than in the cell lines studied. © 1998 Academic Press

The human estrogen receptor (ER[1]) is a member of the superfamily of nuclear steroid receptors. The gene for ER is more than 140 kb in length and contains eight exons. The protein has six functional domains, designated A through F. Domain C, which spans exons 2 and 3, binds DNA and domain E, which spans exons 4–8, contains the hormone binding site (1, 2). The presence of ER in breast tumor cells is considered a good prognosis and the patients who express estrogen receptors in their tumors have an overall longer survival and lower risk of tumor recurrence (3). In addition to its prognostic value, the presence of ER is exploited to treat tumors with antiestrogen therapy (4). Clinical studies have indicated that approximately 50% of patients with ER content >1000 pM/mg protein are found to respond favorably to antiestrogen therapy. Currently, the ER status in breast tumor tissues is determined from rough estimates yielded by microscopically scoring slides subjected to immunohistochemistry techniques. In recent years, due to increased awareness and periodic screening procedures, tumors of small sizes can be detected. In these cases the limited amount of tumor tissue often restricts ER protein quantitation. Several studies have suggested that molecular biological procedures may be preferred for ER quantitation since they require much smaller amounts of tumor tissue. This led to the development of PCR-based methods for the detection and relative quantita-

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[1] Abbreviations used: ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse-transcription polymerase chain reaction.

0003-2697/98 $25.00

tion of ER mRNA in tumor samples (5–7) in recent years. However, none of the methods described thus far can estimate the absolute number of copies of the receptor mRNA.

In this report, we describe a significantly improved PCR approach with which one can quantitate the exact number of ER mRNA copy numbers of wild-type as well as variant transcripts. As a standard of reference for the ER transcripts, we also describe the quantitation of the mRNA copy number of a constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The significance of our PCR template competition assay, therefore, is twofold: (i) survival prognosis and hormone therapy response may be more readily predictable and (ii) the relationship of ER variants to breast cancer growth can be assessed.

MATERIALS AND METHODS

RNeasy midi kits, Ampli*Taq* PCR core kits, and QIAquick gel extraction kits were from QIAGEN Inc. (Santa Clara, CA). Reverse transcriptase kits were from Applied Biosystems. [α-^{32}P]dCTP (sp act 3000 Ci/mmol, Cat. No. AA0005) was from Amersham. A plasmid containing the full-length ER cDNA gene pIC-ER-F was obtained from ATCC. Trizol reagent for total RNA isolation was from Life Technologies. PCR quality water and Tris–EDTA buffer were from Biofluids (Rockville, MD).

Cell Lines and Breast Tumor Samples

The ER positive breast cancer cell lines MCF-7, T47D, and ZR-75, and an ER negative cell line, LCC6, were kind gifts from Dr. Robert Clark, Georgetown University School of Medicine. They were maintained in 90% IMEM without gentamycin and 10% fetal bovine serum. The breast tumor samples were derived from a breast tumor bank established by one of us (I.P.) at Howard University Medical School.

RNA Isolation and Reverse Transcription

Total RNA from breast cancer cell lines was isolated using RNeasy midi kits. This method yielded about 0.8–1.0 mg of total RNA per 10^7 tissue culture cells. Total RNA from powdered breast tumor samples was isolated using Trizol reagent. This method yielded about 30 μg of total RNA per 100 mg of tumor tissue. The isolated RNA was reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase and random hexamers.

PCR

For ER cDNA amplification, the sequences between exons 4 and 8 were amplified using the sense primer 5′-GCCCGCTCATGATCAAACGC-3′ (exon 4, bp 1112–1132) and an antisense primer 5′-TACTTTTGCAAGGAATGCGA-3′ (exon 8, bp 1978–1958) (8). The amplification was carried out in a 12.5-μl reaction volume containing the reverse-transcribed cDNA, 1× PCR buffer, 1× Q solution (Qiagen), 200 μM each of dNTPs, 2 μM each of sense and antisense primers, and 0.6 U of *Taq* polymerase. The PCR conditions were initial denaturation for 5 min at 95°C followed by 94°C for 1 min, annealing for 1 min at 55°C, and extension for 2 min at 72°C for 40 cycles and final extension for 10 min at 72°C. The GAPDH cDNA was amplified using a sense primer, 5′-AAGGCTGAGAACGGGAAGCTTGTCATCAAT-3′ (exon 3, bp 241–270), and an antisense primer, 5′-TTCCCGTCTAGCTCAGGGATGACCTTGCCC-3′ (exon 7, bp 740–711) (9, 10), under the same PCR conditions as described for ER.

Design and Construction of Competitor Template DNAs for the Quantitation of ER and GAPDH by Template Competition

In the template competition approach (11), two templates which can be amplified by the same primers are used in the PCR. If two templates are amplified at the same efficiency, the ratio of their PCR products will reflect the starting ratio of the two template DNAs. This means that if the amount of a competitor DNA is known, then the amount of unknown template can be precisely determined by measuring the two PCR products. This requires that the products generated by two different templates must be distinguishable from each other. For this purpose, a competitor plasmid DNA which contained an extra fragment of DNA internal to the PCR primer location that generates a larger PCR product was prepared.

Competitor template DNA for GAPDH. The competitor plasmid for GAPDH was constructed in two steps. First a 500-bp portion of the GAPDH cDNA was amplified by RT-PCR using RNA extracted from the breast cancer cell line MCF-7. The primers used had an attached upstream *Eco*RI restriction enzyme site and a downstream *Bam*HI restriction enzyme site. The sense and antisense primers were 5′-CCCGAATTCAAGGCTGAGAACGGGAAGCTT-3′ (exon 3, bp 241–261) and 5′-CCCGGATCCTTCCCGTCTAGCTCAGGGATG-3′ (exon 7, bp 740–720), respectively (10). This fragment was subcloned into the *Eco*RI and *Bam*HI sites of pBluescript II SK+ (Stratagene). The resulting plasmid was designated as pBS-GAPDH. Next, a 210-bp fragment of DNA was amplified from bacteriophage λ DNA (from bases 1920 to 2130) (12) by PCR using primers which had attached *Nco*I restriction enzyme sites to both ends. The sense and antisense primers were 5′-CCCCCATGGCTGGACCGCTACGAAATGCGC-3′ (bp 1920–1940) and 5′-CCCCCATGGCGTTCAACAATGGTCGGG-3′ (bp 2130–2110), re-

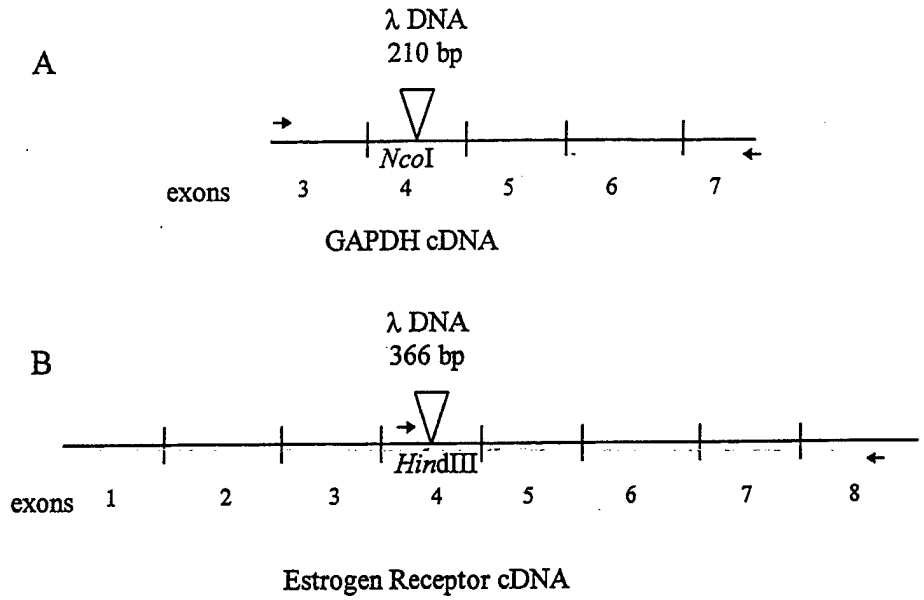

FIG. 1. Design of competitor plasmids for the quantitation of GAPDH and ER copy numbers. (A) pBS-GAPDH-λ. The approximate primer locations are indicated by arrows. A 210-bp λ DNA fragment was inserted into the *Nco*I site in exon 4 of the GAPDH cDNA gene. The resulting GAPDH competitor cDNA generated a 710-bp product by PCR amplification. (B) pSG-ER-λ. The approximate locations of the primers used in exons 4 and 8 are indicated with arrows. A 366-bp λ DNA fragment was inserted at *the Hin*dIII site in exon 4 of the ER cDNA. The resulting ER competitor cDNA generated a 1233-bp product by PCR amplification.

spectively. This fragment was then subcloned into the unique *Nco*I site within the 500-bp piece of pBS-GAPDH. The competitor thus generated was termed as pBS-GAPDH-λ. The schematic representation of the competitor design for GAPDH is shown in Fig. 1A. When pBS-GAPDH-λ was used in a PCR, it generated a fragment 210 bp larger than pBS-GAPDH (not shown).

Competitor template DNA for ER. The competitor plasmid for ER was prepared by inserting λ sequences into an unique *Hin*dIII site in exon 4 of ER cDNA. This construction was also performed in two steps. First, since the commercially procured pIC vector in which ER was cloned (13) contains an additional *Hin*dIII site, the ER cDNA gene was subcloned from pIC-ER-F into the *Eco*RI site of the plasmid pSG5 (Stratagene). Second, a fragment of DNA was amplified from bacteriophage λ DNA using the sense primer 5′-GGGAAGCTTAAACCATTCT-TCATAATTCAA-3′ (bp 37101–37121) containing a 5′ *Hin*dIII linker and the antisense primer 5′-CGCACCAA-CAGGCTCCAAGCC-3′ (bp 37465–37485) which flanked a native *Hin*dIII site in λ (12). This fragment generated a 366-bp DNA when digested with *Hin*dIII. The 366-bp fragment generated above was inserted into the *Hin*dIII site in exon 4 of ER. The competitor thus generated is termed pSG-ER-λ. The schematic representation of the design of the competitor for ER is shown in Fig. 1B. As shown in the figure, the primers used for amplification of ER flank the unique *Hin*dIII site within exon 4. When this cDNA was used as a competitor template in a PCR with the ER primer pair which can amplify exons 4–8, it generated a product that is 366 bp larger than the normal product and thus distinguishable by gel electrophoresis (data not shown).

Competition Assay

The ability of the competition assay to accurately measure ER and GAPDH transcript copy numbers was first confirmed by performing the assay with known amounts of both the competitor templates and the cloned normal GAPDH and ER cDNA genes. The numbers of copies of normal and competitor DNA molecules were determined by multiplying the average mass of a base pair (660 daltons) by the number of base pairs and then dividing Avagadro's number by that value as in the following example for a 3500-bp DNA molecule: $(6.023 \times 10^{23}$ molecules/mol)/3500 bp × 660 g/mol/bp = 2.61×10^{17} molecules/g = 2.61×10^{11} molecules/μg. For these experiments the number of copies of ER cDNA and GAPDH cDNA was kept constant at 10^7 while the concentrations of the competitor DNAs ranged from 10^4 to 10^8 copies. In both ER and GAPDH competition assays, we expect the normal and the competitor plasmid DNAs to cross over at equimolar concentrations (10^7 copies). It was found that the amounts of competitors needed to generate equal ratios of the normal and the competitor PCR products for GAPDH and ER were 10^7 and $10^{7.25}$, respectively. They crossed over as expected or within the range of 1.5–2 times (data not shown), which agrees with the previously

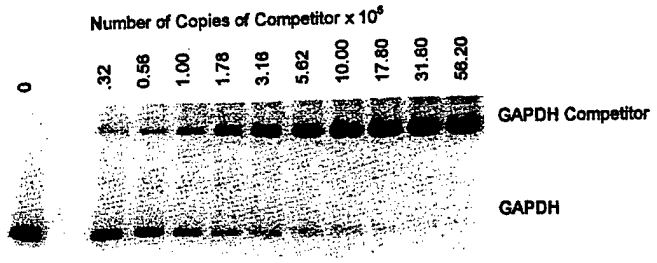

FIG. 2. Template competition assay for GAPDH quantitation in the T47D cell line. PCR amplification with GAPDH specific primers and a constant amount of cDNA prepared by reverse transcription of 50 ng of T47D total RNA was performed in the presence of varying amounts of the competitor pBS-GAPDH-λ. The number of copies of the competitor ranged from 0.32×10^5 (lane 2) to 56.2×10^5 (lane 11) in half-log increments. The PCR amplifications were conducted under the conditions described under Materials and Methods. The amplified products were electrophoresed in 4.5% acrylamide gels and detected by autoradiography.

published reports on the template competition method (11). After verifying the feasibility of the template competition method, we went on to measure the copy numbers of both ER and GAPDH in breast cancer cell lines and tumor samples. To determine ER copy numbers, the cDNA prepared from 250 ng of total RNA was kept constant and the competitor concentration was increased from 10^4 to 10^8 copies in quarter-log dilutions. To determine the number of copies of GAPDH, the cDNA prepared from 50 ng of total RNA was kept constant and the competitor concentration was increased from 10^4 to 10^8 in half-log dilutions. The PCRs were performed as described before.

Detection and Quantitation of PCR Products

For quantitation of the PCR products, [α-^{32}P]dCTP was included in the PCR at 0.5% of the total reaction volume. The radiolabeled PCR products were electrophoresed in 4.5% acrylamide gels, 90 mM Tris–borate, and 0.2 mM EDTA at a 40-mA constant current for 3.5 h. The gels were dried and autoradiographed. The radioactivity in the individual PCR products was quantitated by scanning the autoradiograms in a laser densitometer (Molecular Dynamics).

RESULTS AND DISCUSSION

Clinical studies have suggested that the content of ER in the tumor tissues influences the prognosis and response to antiestrogen therapy in breast cancer (3, 4). Therefore, the ability to precisely quantitate the ER content in the tumor tissues will be of significant benefit to women with breast cancer. Although immunohistochemical and several PCR-based methods have been described, none of them quantify the exact copy numbers of ER in tumor tissues. In the present study we describe a molecular approach to precisely quantitate the ER mRNA copy numbers in the tumor tissues by the template competition method. We have also devised a method to quantitate the exact mRNA copy numbers of a constitutively expressed gene, GAPDH, so that the ER copies from various samples can be expressed with reference to the copy numbers of GAPDH. In addition, we show that the template competition method described here can simultaneously quantitate the copy numbers of ER exon Δ variants. The presence of the variant ERs which have deletions

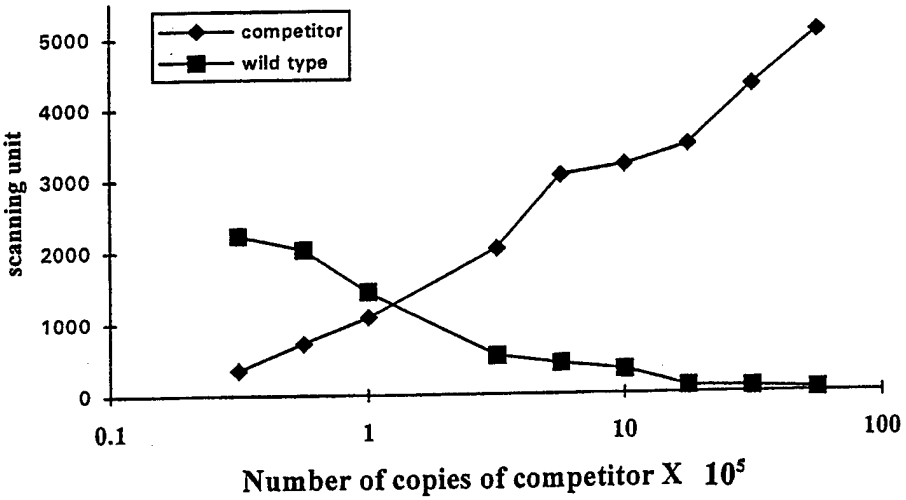

FIG. 3. Absolute quantitation of GAPDH copy numbers in the T47D cell line. The radioactivity in the PCR products of the competition assay was measured by densitometric scanning of the autoradiograms. The normalized scanned units were plotted against the number of copies of the competitor pBS-GAPDH-λ in the PCR. The cross-over point is shown at which the GAPDH products generated from both the competitor and T47D cDNA are equal. The number of copies of GAPDH transcripts is equal to the number of copies of the competitor at the cross-over point.

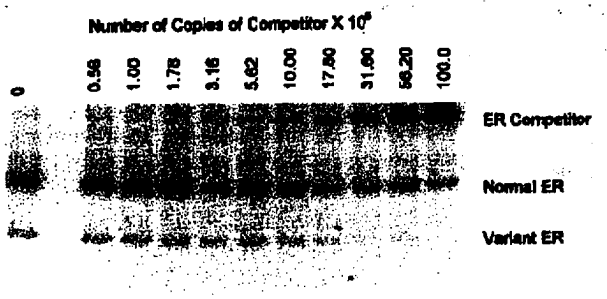

FIG. 4. Template competition assay for ER quantitation in the T47D cell line. PCR amplification with ER specific primers and a constant amount of cDNA prepared by reverse transcription of 250 ng of T47D total RNA was performed in the presence of varying amounts of the competitor pSG-ER-λ. The number of copies of the competitor ranged from 0.58×10^5 (lane 2) to 100×10^5 (lane 11) in quarter-log increments. PCR amplifications were conducted under the conditions described under Materials and Methods. The amplified products were electrophoresed in 4.5% acrylamide gels and detected by autoradiography. Two ER products from cDNA derived from the T47D cell line corresponding to wild-type and exon 7Δ variant are seen and designated as normal ER and variant ER.

in exons 2, 3, 2–3, 5, and 7 in both normal mammary and breast tumor tissues is well documented (14–16).

We designed and cloned the competitor templates which enabled us to quantitate the exact copy number of both GAPDH and ER transcripts by template competition. For ER quantitations, we used the primers which amplified sequences between exons 4 and 8. The primers were first tested on the cDNA prepared by reverse transcription of total RNA from various breast cancer cell lines. We observed the expected 865-bp PCR product plus an additional product size of about 700 bp in MCF-7, T47D, and ZR-75 and the two tumors tested. Neither of these products was observed in cell line LCC6 which is typed as ER negative. Restriction enzyme digestion of the 700-bp fragment suggested that it was the previously characterized exon 7Δ variant and subsequent sequence analysis confirmed its identity (data not shown). After verifying the identity of the 700-bp fragment, we performed the quantitations of GAPDH and ER copy numbers in the ER positive breast cancer cell lines and tumor tissues.

A typical quantitation assay performed for GAPDH using the reverse-transcribed cDNA prepared from the T47D cell line is presented in Figs. 2 and 3. The amount of competitor in the PCR ranged from 0.32×10^5 copies (Fig. 2, lane 2) to 56.2×10^5 copies (Fig. 2, lane 11). As seen in Fig. 2, the GAPDH PCR product from the T47D cell line (lower band) decreased in the presence of increasing amounts of the competitor plasmid pBS-GAPDH-λ (upper band). To determine the exact copy numbers of GAPDH, first the radioactivity in the normal and the competitor PCR products was scanned densitometrically. Second, since the specific activities of the competitor and normal PCR products are different because of their size difference, the scanning units were normalized to the size of the competitor. Third, the normalized scanning units were plotted against the number of copies of the competitor template in the PCR. A representative graph for GAPDH quantitation in the T47D cell line is shown in Fig. 3. As seen in Fig. 3, the cross-over point at which the competitor and the reverse-transcribed cDNA gave equal

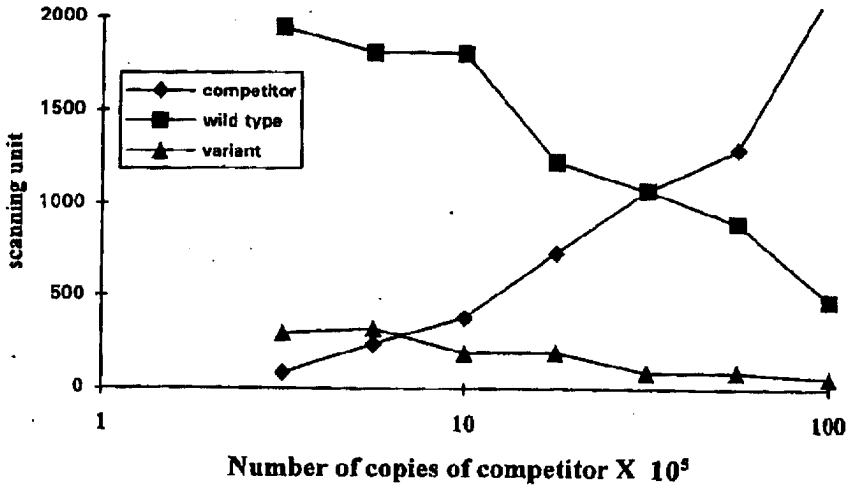

FIG. 5. Absolute quantitation of ER copy numbers in the T47D cell line. The radioactivity in the PCR products of the competition assay was measured by densitometric scanning of the autoradiograms. The normalized scanned units were plotted against the number of copies of the competitor pSG-ER-λ in the PCR. The cross-over points are shown at which the specific ER wild-type and exon 7Δ variant PCR products generated from T47D cDNA and competitor DNA are equal. The number of copies of ER wild-type and variant transcripts is equal to the number of copies of the competitor at the respective cross-over points.

TABLE 1

No.	Cell type/ tumor	Copies of ER mRNA/ 10^5 copies of GAPDH		Ratio of exon 7Δ variant to wild type
		Wild type	Exon 7Δ variant	
1.	T47D	5.0×10^5	1.0×10^5	0.2
2.	MCF-7	4.8×10^4	1.5×10^4	0.31
3.	ZR-75	1.1×10^4	4.9×10^3	0.44
4.	Tumor 1	2.2×10^4	1.5×10^4	0.68
5.	Tumor 2	$<6.0 \times 10^3$	$<6.0 \times 10^3$	~1.00

amounts of PCR products was 1.25×10^5 copies. Therefore, the number of copies of GAPDH in T47D cDNA prepared from 50 ng of total RNA was 1.25×10^5 copies. The number of copies of GAPDH from MCF-7, ZR-75, tumor 1, and tumor 2 was determined by similar procedures. They were 7.3×10^5, 4.3×10^5, 8.1×10^4, and 3.8×10^4, respectively, per 50 ng of reverse-transcribed total RNA. The values in the tumor samples are about 10 times lower than the tissue culture cell lines for the same amount of reverse-transcribed total RNA. The reasons for the lower values in tumor samples are not clear. This observation, however, validates the importance of normalizing the ER values to GAPDH in tumor samples instead of relying only on the RNA concentration.

A typical quantitation assay for the ER wild-type and exon 7Δ variant copy numbers using the reverse-transcribed cDNA prepared from the T47D cell line is presented in Figs. 4 and 5. The amount of competitor used in the PCR ranged from 0.56×10^5 copies (Fig. 4, lane 2) to 100×10^5 copies (Fig. 4, lane 11). As seen in Fig. 4, the ER wild-type and exon 7Δ variant products (middle and lower bands, respectively) from the T47D cell line decreased in the presence of increasing amounts of the competitor plasmid pSG-ER-λ (upper band). To determine the exact copy numbers of ER wild-type and the exon 7Δ variant, the densitometric scans of the radioactivity were normalized as in the case of GAPDH and the normal and the competitor PCR products were plotted against the number of copies of the competitor template in the PCR. A representative graph for the ER wild-type and exon 7Δ variant quantitation in the T47D cell line is shown in Fig. 5. As seen in Fig. 5, the wild-type ER and competitor templates generated equal amounts of PCR products when the competitor concentration was at 31.6×10^5 copies. The exon 7Δ variant and the competitor products crossed over when the competitor concentration was 6.6×10^5 copies. Thus, the numbers of copies of ER wild-type and exon 7Δ variant in T47D cDNA prepared from 250 ng of reverse-transcribed total RNA were 31.6×10^5 and 6.6×10^5, respectively. The numbers of copies of the wild-type and the exon 7Δ variant in MCF-7, ZR-75, and tumor tissues were determined by similar procedures. The numbers of copies of both the wild-type and exon 7Δ variant in all the cell lines and tumor tissues studied were normalized to 10^5 copies of GAPDH. The results are presented in Table 1. The numbers were found to be repeatable in multiple experiments. Our results indicate that the T47D cell line expresses the highest amounts of ER transcripts among the cell lines studied. The results presented in Table 1 also show that the ratios of the variant to wild type are highest in ZR-75 (0.44) and lowest in T47D (0.2) among the cell lines studied. The ratios of the exon 7Δ variant to wild type in the tumor tissues are considerably higher (0.68 and ~1 in tumors 1 and 2, respectively) than in the cell lines studied. The significance of this higher variant to wild-type ratio in tumor tissues is not known. It was previously shown that the exon 7Δ variant forms a heterodimer with the wild-type protein and exerts a dominant negative effect due to constitutive DNA binding activity in the absence of the hormone. It was also suggested that the ratio of variant to wild type influences the response to antiestrogen therapies (19). However, it is not known whether the increase in the copy numbers of this variant is responsible for the genesis and progression of breast cancers.

The results presented in this report clearly establish the feasibility of determining the exact mRNA copy numbers of the ER wild type and variants in tumor tissues. Since the ER competitor we have prepared has the insert in exon 4, it can be used with any set of primer pairs which encompasses exon 4. The quantitation procedures described here will highly facilitate establishing an index between the mRNA copy numbers of ER wild- and variant-type transcripts per a given number of copies of GAPDH transcripts in the tumors and response to antiestrogen therapies.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Cancer Institute (CA 71150), the Susan G. Komen breast cancer foundation (to I.P.), and the Department of Defence (DAMD 17-94-J-4495) (to I.P. and R.E.T.). The technical assistance of Ms. Chung Ling Go and Dr. Zang Li is acknowledged. Dr. Robert Clark is gratefully acknowledged for his gifts of breast cancer cell lines.

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Paris, 20th December 1999

Paper N°: **JSBMB 99-7253**

Dear Dr Poola,

It is a pleasure to inform you that your manuscript entitled :

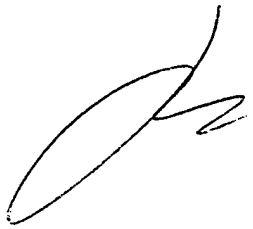
IDENTIFICATION OF TWENTY ALTERNATIVELY SPLICED ESTROGEN ...

by I. POOLA *et al.*

is ready for publication in the *JOURNAL OF STEROID BIOCHEMISTRY & MOLECULAR BIOLOGY*. It is being sent to the printers and you should receive the corresponding proofs directly from Elsevier in due course.

The anticipated issue of publication is : Volume 72, N° 2, 2000.

Yours sincerely,



Dr J. R. PASQUALINI
Editor-in-Chief

IDENTIFICATION OF TWENTY ALTERNATIVELY SPLICED ESTROGEN RECEPTOR ALPHA mRNAs IN BREAST CANCER CELL LINES AND TUMORS USING SPLICE TARGETED PRIMER APPROACH

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Running Title: Analysis of ER splice variants by a novel approach.

ABSTRACT

Estrogen receptor (ER) alpha splice variant transcript profiles were analyzed by RT PCR in six ER positive breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, three ER negative cell lines, MDA-MB-435, MDA-MB-235 and LCC6, and three ER positive malignant breast tumors using targeted primers which specifically anneal to the splice junctions of exon 2Δ, exon 3Δ, exons 2-3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ. The partner primers were chosen such that largest possible transcripts were amplified between exons 1-8. The results described here show that each splice specific primer amplified not only the single exon deleted transcript but also a number of related transcripts that have deletions in various combinations of exons. The exon 2Δ specific primer amplified five transcripts that have deletions in exon 2, exons 2 and 7, exons 2, 5, and 7, exons 2 and 4-5, and exons 2 and 4-6. The exon 3Δ specific primer amplified two transcripts that have deletions in exon 3, and exons 3 and 7. The exon 2-3Δ specific primer amplified three products that have deletions in exons 2-3, exons 2-3 and 7 and exons 2-3, 5 and 7. The exon 4Δ specific primer amplified two products that have deletions in exon 4, and exons 4 and 7. The exon 5Δ specific primer amplified three transcripts, that have deletions in exon 5, exons 5 and 2, and exons 5, and 2-3. The 6Δ specific primer amplified only one transcript that has a deletion in exon 6. The 7Δ specific primer amplified four transcripts, that have deletions in exon 7, exons 7 and 4, exons 7 and 3-4, and exons 7 and 3-5. None of the above splice specific primers amplified the wild type ER sequences. The six ER positive cell lines differed in the patterns of the variant transcripts and among the three ER negative cell lines analyzed, only MDA-MB-435 showed

the presence of exon 2Δ and exon 4Δ transcripts. Analyses in the tumor samples indicated that the above transcripts are extensively modified.

INTRODUCTION

The estrogen receptor (ER) alpha mRNA undergoes alternate splicing, generating transcripts containing single, double or multiple exon deletions. The presence of ERα transcripts with deletions in exons 2-, 3-, 2-3, 2-5, 4-, 5-, 6- and 7 has been described in breast cancer cell lines and normal- and malignant breast tissue samples [1-4]. Although the exact function(s) of these splice variants is not established, it has been hypothesized that the splice variant mRNAs may result in proteins that differ in activity. These may differentially modulate the ER signalling pathway in normal tissues. Consequently, changes in the balance of these transcripts could perturb the ER signaling pathway and contribute to tumor progression. Several studies suggested that the expression of certain exon deletion transcripts is deregulated during breast tumorigenesis. It was shown that the exon 5 deletion transcript was significantly elevated in ER^- PgR^+ breast tumor tissues [5]. Elevated levels of exon 7 splice transcripts have also been reported in $ER^+/PR^-/pS2-$ compared to ER^+/PgR^+ tumors [6]. It has been reported that expression of the exon 3- deleted mRNA is reduced in breast tumor tissue compared with normal tissue [7]. Differential expression of exon 5 and exon 7 deletion transcripts also seem to influence the estrogen responsiveness in breast cancer cell lines [8]. All these reports suggest that expression of some ER variants is altered in human breast tumors and may contribute to tumorigenesis, tumor progression and response to hormones. Therefore, it is important to qualitatively and quantitatively investigate the levels and pattern of ER splice variant expression between normal and neoplastic tissues, and amongst groups of tumors with different characteristics. Yet there are no specific methods available which can

precisely detect and quantify the alternatively spliced ER molecules.

Conventionally, the ER exon deletion variant transcripts are characterized by co-amplification with the wild type sequences using reverse transcription polymerase chain reaction (RT-PCR) approaches which by virtue of specific primer design are focussed on small regions of the known wild type mRNA. However, there are several practical limitations to this approach. Firstly, the threshold of detection- since the wild type transcripts are present in large excess to alternatively spliced molecules, and a competitive amplification occurs amongst the wild type and all the alternatively spliced transcripts. Detection of products corresponding to alternatively spliced molecules depends upon the relative expression levels of their mRNA species with in the sample. Thus, spliced transcripts expressed at low levels may fall below the threshold of detection. Secondly, this approach cannot distinguish those mRNAs with multiple deletions in distant exons. For example, an ER transcript which has deletions in exons 2- and 7 cannot be distinguished from transcripts having single deletions in exon 2 or exon 7 by this method, and finally transcripts with similar sized deletions cannot be distinguished by gel exclusion chromatography.

To circumvent all the above described limitations, we have developed a new approach to characterize the alternatively spliced molecules. This involves the targeted amplification of the alternatively spliced molecules as separate gene populations with-out co-amplification of wild type molecules using specific primers designed for the alternative splice junctions [9]. In the current study, we analyzed the ER single,- double,- and multiple exon deletion variant

transcripts in breast cancer cell lines and tumors by RT PCR using the splice targeted primers. We show here that each splice specific primer amplifies not only the single exon deleted transcript but also a number of related transcripts with deletions in various combinations of exons. Our results also show that several alternatively spliced molecules are either missing or extensively modified in tumor samples.

MATERIALS AND METHODS

AmpliTaq PCR core kits and QIAquick gel extraction kits were obtained from QIAGEN Inc., Santa Clara, CA. All the primers used in the current study were synthesized by Gibco-BRL Life Technologies. Reverse transcriptase kits were purchased from Applied Biosystems. The pCR®II-TOPO cloning vector was obtained from Invitrogen. PCR quality water and Tris-EDTA buffer were from Biofluids, Rockville, Md. The total RNA samples from breast cancer cell lines and tumors were prepared using Trizol reagent (Gibco-BRL). The integrity of all the RNA preparations was confirmed by electrophoresis and ethidium bromide staining and amplification of the constitutively expressed gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH). The ER status of all the tumors used in the current study was determined immunohistochemically by Oncotech laboratories using monoclonal antibodies against the NH_2-terminal (A/B region) of the receptor.

Targeted primers for the amplification of single- double and multiple exon deletion variant cDNAs of ER. We have previously shown that the primers targeted at the

alternate splice junctions that have a minimum of 3 out of 4 unique bases at the extreme 3' end will specifically amplify the spliced junction without amplifying the flanking wild type exons and in order to design such a primer, the overhang sequences can extend up to eight bases past the splice junction [9]. The splice specific primers used in the current study were designed based on these principles. The splice specific primers used for amplifying 2Δ, 3Δ, 2-3Δ, 4Δ, 5Δ, 6Δ, and 7Δ were ER SX1/3, 5' CGCCGGCATTCTACAG 1/3 GACAT 3' (positions, exon 1, bp 669-684, and exon 3, bp 876-880), ER SX2/4, 5' AAGAGAAGTATTCAAG 2/4 GGATA 3' (positions, exon 2, bp 860-875 and exon 4, bp 993-997), ER SX1/4, 5' GCCGGCATTCTACAG 1/4 GGATAC 3' (positions, exon 1, bp 670-684 and exon 4, bp 993-998), ER SX3/5, 5' GTGGGAATGATGAAAGGTG 3/5 GCTTT 3' (positions, exon 3, bp 974-992 and exon 5, bp 1329-1333), ER AX4/6, 5' ATTTTCCCTGGTTC 6/4 CTGGCAC 3' (positions, exon 6, bp 1481-1468 and exon 4, bp 1328-1322), ER AX 5/7, 5' CAGAAATGTGTACACTC 7/5 CTGT 3' (positions, exon 7, bp 1618-1603 and exon 5, bp 1468-1465) and ER AX6/8, 5' CTCCATGCCTTTGTTA 8/6 CAGAA 3' (positions, exon 8, bp 1801 to 1786 and exon 6, bp 1601-1597) respectively. The partner primer for 2Δ, 3Δ, 2-3Δ, and 4Δ splice specific primers was ERA, 5' GCACTTCATGCTGTACAGATGC 3' (position, exon 8, bp 1822-1801) and for 5Δ, 6Δ, and 7Δ primers was ERS, 5' TGCCCTACTACCTGGAGAACG 3' (position, exon 1, bp 615-635). The sequence and locations of all the primers described here are based on the full length ER cDNA sequence published by Green and others [10].

Reverse Transcription and PCR. The total RNA was reverse transcribed to cDNA

using Maloney Murine Leukemia Virus reverse transcriptase and random hexamers. Briefly, the standard reaction mixture contained 1 μg of total RNA, 2.5 units of MuLV reverse transcriptase, 1 mM each of dNTPs, 2.5 μM random hexamers, 1 U of RNAse inhibitor, 5 mM $MgCl_2$ and 1 X PCR buffer in a total volume of 20 μl. To reverse transcribe the RNA, the reaction tubes were first left at room temperature for 10 min, followed by incubations at 42^0 C for 15 min, 99^0 C for 5 min and finally 5^0 C for 5 min. The Polymerase Chain Reactions were performed in an automatic thermal cycler (MJ Research) as described previously [11] in a 25 μl reaction volume containing the cDNA reverse transcribed from 250 ng of total RNA, 1 X PCR buffer, 1 X Q solution, 200 μM each of dNTPs, 2 μM each of sense and anti-sense primers and 0.6 U of Taq polymerase. The GAPDH was amplified using a sense primer, 5' AAGGCTGAGAACGGGAAGCTTGTCATCAAT 3' (position, exon 3, bp 241-270), an anti-sense primer, 5' TTCCCGTCTAGCTCAGGGATGACCTTGCCC 3' (position, exon 7, bp 740 -711) (12) and cDNAs prepared from reverse transcription of 25 ng of total RNA. To amplify the exon deletion variant cDNAs in the tumor samples, PCRs were performed using cDNAs prepared from reverse transcription of 500-750 ng of total RNA. The PCR conditions were initial denaturation for 1 min at 95^0 C followed by 94^0 C for 1 min, annealing for 1 min at the specified temperature depending on the primer pair used, extension for 2 min at 72^0 C for 40 cycles and final extension for 10 min at 72^0 C. The annealing temperature for 2Δ, 2-3Δ, 4Δ and 6Δ specific primers was at 61^0 C, for 3Δ and 7Δ primers was 55^0 C and for 5Δ specific primer was 65^0 C.

Detection and sequence analysis of PCR products. To detect the PCR amplified

ER splice variant products from cell lines, an aliquot (4-7 µl) was electrophoresed in 1% agarose gels in Tris-acetate EDTA buffer and detected by ethidium bromide staining. To detect the PCR products of GAPDH, 1 µl was electrophoresed and the ER splice variant products amplified from tumor samples, 12-25 µl of the products were analyzed on the gel. To determine the identity of the PCR amplified ER splice variant products, they were electrophoresed in 1.2% agarose gels and purified individually using the QIAquick gel extraction kit. The purified products were cloned into pCR®II-TOPO vector and sequenced by cycle sequencing method on an automated DNA sequencer (carried out at the Biopolymer Laboratory, University of Maryland School of Medicine, Baltimore, MD).

RESULTS

We analyzed the ER single-, double-, and multiple exon deletion transcripts by RT PCR using primers targeted at the splice junctions of exon 2Δ, exon 3Δ, exons 2-3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ. The partner primers were chosen such that largest possible transcripts were amplified between the exons 1-8. This permitted the amplification of not only the single exon deletion transcripts but also those with multiple deletions in distant exons. The PCR analyses were carried out in six ER positive breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, and LCC9 and three ER negative cell lines, MDA-MB-435, MDA-MB-235 and LCC6. Three ER positive breast tumor samples were also included to test the applicability of splice targeted primer approach in analyzing the above transcripts in clinical samples. The results described here on the analysis of various alternatively spliced ER

transcripts were repeatable in several experimental trials.

Analysis of exon 2Δ transcripts. The exon 2Δ transcript profiles in seven cell lines and three tumors are shown in Figure 1A. The lanes M1 and M2 contain Gibco-BRL 1 kb- and 100 bp ladders respectively. The ER positive cell lines, MCF-7, ZR-75, LCC1, LCC2, and LCC9, amplified three major bands of sizes of about 960 bp, 780 bp and 640 bp. The cell line T47D did not amplify the 960 band, instead it amplified two products which are higher than 960 bp. All six ER positive cell lines amplified several minor bands ranging from 480-330 bp. Unexpectedly, one of the three ER negative cell lines tested, MDA-MB-435, also amplified 960-, 640- and 480 bp bands and three additional bands that showed lower mobility than the 960 bp band. Tumor 3 did not amplify any product. Tumor 2 amplified minor bands at 640- and 480 bp and tumor 1 amplified only the 480 bp as a minor band. To determine the identify of the above products, the PCR products from LCC1 cells were cloned and sequenced. The 960-, 780-, 640-, 480- and 330 bp products were identified as ER transcripts with deletions in exon 2, exons 2 and 7, exons 2, 5, and 7, exons 2 and 4-5, and exons 2 and 4-6, respectively (Figure 1B). It was also found that the exons 2Δ and 4-6Δ product had 20 bps missing in exon 7. Figure 1A also shows the expression levels of GAPDH in the above cell lines and tumors.

Analysis of exon 3Δ transcripts. The exon 3Δ transcript profiles in seven cell lines and three tumors are shown in Figure 2A. Lanes M1 contain Gibco-BRL 100 bp ladders. The ER positive cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, amplified two

products of sizes about 845 bp and 661 bp. The ER negative cell lines and two of the tumors in the study did not amplify these two bands. Only one of the three tumors (Tumor 5) amplified the 845 bp but not 661 bp product. To determine the identity of the above products, the PCR products from LCC1 cell line were cloned and sequenced. The 845-, and 661 bp products were identified as ER transcripts that have deletions in exon 3, and exons 3 and 7 respectively (Figure 2B).

Analysis of exons 2-3Δ transcripts. The PCR product profiles of exon 2-3Δ transcripts in seven cell lines and three tumors are shown in Figure 3A. The lanes M1 and M2 contain Gibco-BRL 1 kb- and 100 bp ladders respectively. All the six ER positive cell lines amplified three products with approximate sizes of 840 bp, 660 bp and 520 bp. Two minor bands between 840 bp and 660 bp are also seen. One of the three ER negative cell line, MDA-MB-435, generated a minor product slightly bigger than the 840 bp product. To determine the identities of 840-, 660- and 520 bp products, the PCR products from LCC1 were cloned and sequenced. The 840-, 660-, and 520 bp products were identified as ER transcripts with deletions in exons 2-3, exons 2-3 and 7, and exons 2-3, 5 and 7 respectively (Figure 3B). Tumor 1 generated three bands of which two corresponded to exons 2-3Δ, and exons 2-3Δ and 7Δ. The third band showed slightly higher mobility than the exons 2-3Δ, 5Δ and 7Δ product. Tumor 2 amplified two bands of approximate sizes 700 bp and 550 bp which are slightly higher than the exons 2-3Δ and 7Δ, and exons 2-3Δ, 5Δ and 7Δ products. The third tumor generated only the exons 2-3Δ and 7Δ product.

Analysis of exon 4Δ transcripts. The exon 4Δ transcript profiles in seven cell lines and three tumors are shown in Figure 4A. The lane M1 contains Gibco-BRL 100 bp ladder. The ER positive cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, amplified two products of sizes of about 512 bp, and bp 328 bp. One of the three ER negative cell lines, MDA-MB-435, also amplified faint bands of 512 and 328 bps. All three of the tumors tested amplified these two products. To identify the above products, the PCR products from LCC9 cell line were cloned and sequenced. The 512-, and 328 bp products were identified as ER transcripts with deletions in exon 4, and exons 4 and 7 respectively (Figure 4B).

Analysis of exon 5Δ transcripts. The profiles of exon 5Δ transcripts in seven cell lines and three tumors are shown in Figure 5A. The lanes M1 and M2 contain Gibco-BRL 1 kb- and 100 bp ladders respectively. All the ER positive breast cancer cell lines except MCF-7 amplified one major product and two minor products of approximate sizes, 730 bp, 540 bp and 420 bp respectively. The MCF-7 and all the three ER negative cell lines did not generate any products. To determine the identity of 730-, 540- and 420 bp products, the PCR products from ZR-75 were cloned and sequenced. The 730-, 540- and 420 bp products were identified as ER transcripts having deletions in exon 5, exons 5 and 2, and exons 5 and 2-3 respectively (Figure 5B). The three tumor samples analyzed gave very distinct products. Tumor 1 amplified all the above three products and an additional product between exon 5Δ and the exons 5Δ and 2Δ products. Tumor 2 amplified one product between exon 5Δ and exons 5Δ and 2Δ products similar to tumor 1 and two products of approximate sizes 500 bp and 350 bp. Tumor 3 amplified only the 500 bp and 350 bp products. Neither tumor 2 or 3 amplified

the major single deletion product.

Analysis of exon 6Δ transcripts. The profiles of exon 6Δ transcripts in seven cell lines and three tumors are shown in Figure 6A. The lanes M1 and M2 contain Gibco-BRL 1 kb- and 100 bp ladders respectively. All ER positive breast cancer cell lines amplified one major product of approximate size, 866 bp. It was identified as the transcript that has a deletion in exon 6 (Figure 6B). None of the ER negative cell lines amplified any product. We could not detect any double or multiple deletion transcripts with 6Δ primer. The three tumors analyzed did not amplify any products (Figure 6A).

Analysis of exon 7Δ transcripts. The profiles of exon 7Δ cDNAs in seven cell lines and three tumors are shown in Figure 7A. The lanes M1 and M2 contain Gibco-BRL 1 kb- and 100 bp ladders respectively. All the six ER positive breast cancer cell lines generated a major 1 kb band and a minor band of approximately 665 bp. The cell line LCC2 generated an additional two minor bands of sizes 560 bp and 410 bp. The cell line LCC1 also generated 560 bp minor band and LCC9 generated the 410 bp minor band. In all these cell lines, several closely spaced minor bands were visualized between 1 kb- and 665 bp products. To determine the identities of 1kb-, 665-, 560- and 410 bp products, the PCR products from LCC1 were cloned and sequenced. They were identified as ER transcripts with deletions in exon 7, exons 7 and 4, exons 7 and 3-4, and exons 7 and 3-5 respectively (Figure 7B). The three tumor samples analyzed gave very distinct products. Tumor 1 amplified all the above four products, similar to LCC1 cell line. However, the exons 7Δ and 4Δ product is seen as a major band and

the single deletion 1000 bp product as a minor band. Tumor 2 gave a similar profile to tumor 1, and tumor 3 did not amplify any product. Tumor 3 was previously shown not to have any exon 7Δ transcript when analyzed by co-amplification with wild type sequences between exon 4-8 [13].

DISCUSSION

In the current study we applied a novel approach to specifically amplify a particular category of alternatively spliced ER molecules, from a pool of other alternatively spliced- and wild type ER genes, using primers which anneal to the spliced junctions. We used primers targeted at the splice junctions of exon 2Δ, exon 3Δ, exons 2-3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ transcripts. The results described above on the identities of various transcripts, amplified by the seven splice specific primers, are summarized in Table 1. Each splice specific primer amplified not only the single exon deleted transcript but also a number of related cDNAs that have deletions in various combinations of exons. None of the splice specific primers amplified the wild type ER sequences. The seven specific primers amplified a total of twenty transcripts, of which fourteen had double or multiple exon deletions. Although single,- a few double,- and multiple deletion variants have been described, most of the double and multiple deletion transcripts described here were not previously reported.

Our results show that ten of the twenty transcripts identified have exon 7 deletion,

suggesting that this is the most frequently deleted exon. Examination of the products amplified by exon 2Δ, exon 3Δ, and exon 4Δ specific primers indicated a trend in the deletion of exons. In all these cases the double deletion transcript identified had the deletion of exon 7 (Figures 1B, 2B, and 4B). A similar trend was seen for the exons 2-3Δ primer amplified products (Figure 3B). These results suggest that initial deletion of a particular exon is mostly followed by the deletion of exon 7. Interestingly, the exon 7Δ specific primer recognized only one of the double deletion products, the exons 7Δ and 4Δ (Figure 7B). This preferential amplification may be due to competition among various transcripts. The detection of double deletion transcripts, the exons 5Δ and 7Δ, and exons 6Δ and 7Δ, was not possible in our studies because of the 5Δ and 6Δ specific primers design. The data presented here also show that the third largest cDNA amplified by 2Δ and exons 2-3Δ specific primers had the deletion of exon 5 suggesting that the third most common exon to be deleted in a transcript after the deletion of exon 7 is the exon 5. These observations also indicate that alternative splicing of the ER transcript takes place in a sequential manner, rather than at random. The 3Δ targeted primer did not amplify the triple deletion transcript, which lacked exons 3, 5, and 7 in our studies, probably due to its low abundance. The 4Δ primer did not amplify because of its unique design.

Among the seven targeted primers tested, only 2Δ and 7Δ primers amplified the transcripts with deletions in consecutive exons (Figures 1B and 7B respectively and the Table). The profile of these transcripts suggests that after the deletion of exon 2 in a transcript, if the second deletion is initiated at exon 4, the deletions seem to proceed up to

exon 5 or 6. Similarly, after exon 7 deletion, if the second deletion is initiated at exon 3, the deletions seem to proceed up to exon 4 or 5. Examination of the other multiple deletion transcripts indicated that none of those had single exon 3Δ, instead the deletion of exon 3 appears to be associated with either exon 2 or exon 4 deletion (Figures 3B and 7B respectively).

The results presented in Figures 1-7 show some differences between estrogen-dependent and independent ER positive cell lines in the patterns of variant transcripts. The LCC1, LCC2, and LCC9 are estrogen-independent cell lines derived from the estrogen-sensitive parent cell line, MCF-7, after exposure to steroidal (ICI 182, 780)- or non-steroidal (Tamoxifen) anti-estrogens [14, 15]. These three cell lines did not show any differences in variant expression, suggesting that no ER remodeling is associated with either acquired Tamoxifen [14] or Tamoxifen and ICI 182,780 crossresistance [15]. In contrast, there seems to be some differences in ER variant expression associated with acquired estrogen-independence in these cells. For example, all three of the estrogen-independent cells contain the exons 7Δ, and 3-4Δ and exons 7Δ, and 3-5Δ transcripts. These are absent in the parental MCF-7 cells, and in the T47D and ZR-75 cells. Loss of exon 7 might be expected to affect ligand binding as might deletion of exon 5 and possibly exon 4. The entire hinge region would be lost in the 3-4Δ and 3-5Δ containing transcripts. Elimination of the ligand binding domain and part of the hinge region can produce transcriptionally active protein [16], overexpression of which could contribute to estrogen-independence. While expression of the exons 7Δ, and 3-4Δ and exons 7Δ, and 3-5Δ transcripts is associated with acquired estrogen-

independence, their function and whether significant amounts of these proteins are made, remain unclear. Another major difference observed is the absence of exon 5Δ, exons 5Δ and 2Δ and exons 5Δ, and 2-3Δ transcripts in the parental MCF-7 cells (Figure 5A). It is possible that, these cells are estrogen-dependent, in part, because of the absence of 5Δ transcript, which was reported to possess ligand independent transcriptional property. However, absence of 5Δ transcript alone may not determine the estrogen-dependency because this transcript is detected in both T47D and ZR-75. It is possible that several splice variants, and their relative amounts to the wild type alpha receptor and the amounts of beta receptor in a given cell may influence estrogen-dependency rather than a single transcript.

The exon deletion transcript analysis in tumor samples showed very interesting findings. In the cell lines, the most abundant product each specific primer amplified was the single deletion product and the second most abundant product was the double exon deleted transcript in the case of exon 2Δ, exon 3Δ, exon 4Δ, exon 5Δ and exon 7Δ. In the case of exons 2-3Δ specific primer, they are double- and triple exon deleted transcripts. However, different primers gave different results in tumor samples. When three tumors were analyzed with exon 7Δ specific primer, two tumors showed the presence of four transcripts similar to the cell lines. However, the ratio of each transcript appears to be different compared to the cell lines. In the case of exon 2Δ transcripts, only two tumors showed the presence of minor bands and none of them amplified the single- or double deletion products. When analyzed for the exons 2-3Δ containing transcripts, only one of the tumors generated 2-3Δ product, and the other two amplified the multiple deletion products, that appear to have other modifications,

such as base pair insertions/deletions (Figure 3A). Similar observations were made when analyzed for exon 5Δ transcripts (Figure 5A). In summary, 5Δ and 2-3Δ transcripts are altered for base pair deletions and alterations, 2Δ, 3Δ and 6Δ transcripts are mostly absent, 7Δ transcript ratios are altered and 4Δ transcripts are unchanged in the tumor samples. These results suggest that the patterns and levels of ER variants undergo extensive alterations in tumor tissues.

The results presented in the current study clearly demonstrate the efficacy of the novel approach for analyzing the ER splice variant transcripts in the cell lines and tissue samples using targeted primers designed at alternate splice junctions. We believe that the new approach described here will be useful in 1) delineating the functional roles of ER exon deletion variants in estrogen induced signal transduction processes, 2) analyzing the changes in the profiles of splice variants in the tumor tissues compared to normal tissues, 3) evaluating their role in tumorigenesis, tumor progression and loss of hormone dependency, 4) predicting prognosis and response to anti-hormone therapy and finally 5) developing tissue specific synthetic estrogens and anti-estrogens.

ACKNOWLEDGEMENTS

This work was supported by grants from the Susan G. Komen breast cancer foundation and the Department of Defence (DAMD 17-94-J-4495) to I.P.

ABBREVIATIONS

ER, estrogen receptor, PgR, Progesterone receptor, GAPDH, Glyceraldehyde 3-phosphate dehydrogenase, Exon Δ, Exon deletion; AX, anti-sense; and SX, sense.

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FIGURE LEGENDS

Figure 1. Analysis of ER exon 2Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 2Δ specific primer. The ER exon 2Δ transcripts were analyzed using the specific sense primer, ER SX1/3, and an anti-sense primer ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. **Panel A** shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the Gibco-BRL 1 kb- and 100 bp ladders respectively. The GAPDH profile in all the above samples is also shown. **Panel B** illustrates the identity of the PCR products as determined by sequence analysis. Lanes M have 100 bp ladders.

Figure 2. Analysis of ER exon 3Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 3Δ specific primer. The ER exon 3Δ transcripts were analyzed using ER SX2/4 and ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. **Panel A** shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 4, 5, and 6. Lanes M1 contain the Gibco-BRL 100 bp ladders. **Panel B** illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the 100 bp ladder.

Figure 3. Analysis of exons 2-3Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 2-3Δ specific primer. The exon 2-3Δ transcripts were analyzed using ER SX1/4 and ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. In both A and B panels, lanes M1 and M2 contain the Gibco-BRL 1 kb- and 100 bp ladders respectively. **Panel A** shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. **Panel B** illustrates the identity of the PCR products as determined by sequence analysis.

Figure 4. Analysis of ER exon 4Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 4Δ specific primer. The ER exon 4Δ transcripts were analyzed using ER SX3/5 and ERA. To determine the identity of various PCR products, the products from LCC9 were cloned and sequenced. **Panel A** shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 4, 5, and 6. Lanes M1 contain the Gibco-BRL 100 bp ladders. **Panel B** illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the 100 bp ladder.

Figure 5. Analysis of exon 5Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 5Δ specific primer. The exon 5Δ transcripts were analyzed using ER AX4/6 and a sense primer ERS. To determine the identity of various PCR products, the products from ZR-75 were cloned and sequenced. **Panel A** shows the PCR products

amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the GiBco-BRL 1 kb- and 100 bp ladders respectively. **Panel B** illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

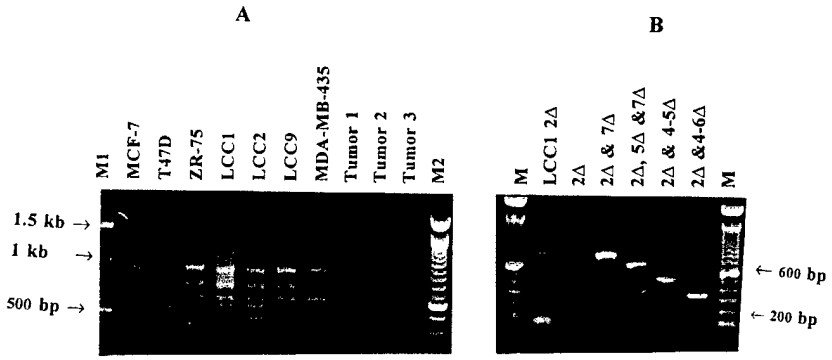
Figure 6. Analysis of exon 6Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 6Δ specific primer. The exon 6Δ transcripts were analyzed using ER AX5/7 and ERS. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. **Panel A** shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 4, 5, and 6. Lanes M1 and M2 contain the Gibco-BRL 1 kb- and 100 bp ladders respectively. **Panel B** illustrates the identity of the PCR product as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

Figure 7. Analysis of exon 7Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 7Δ specific primer. The exon 7Δ transcripts were analyzed using ER AX6/8 and ERS. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. **Panel A** shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the Gibco-BRL 1 kb- and 100 bp ladders respectively. **Panel B** illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

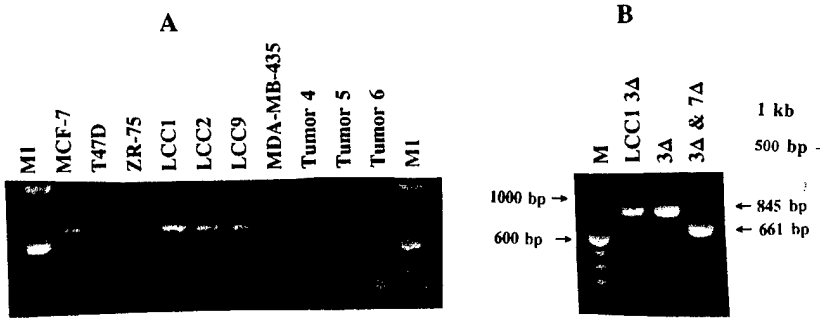
Table.1

# Splice Specific Primer	cDNAs Amplified
1. ER SX1/3	2Δ, 2Δ & 7Δ, 2Δ, 5Δ & 7Δ, 2Δ & 4-5Δ and 2Δ & 4-6Δ
2. ER SX2/4	3Δ and 3Δ & 7Δ
3. ER SX1/4	2-3Δ, 2-3Δ & 7Δ and 2-3Δ, 5Δ & 7Δ
4. ER SX3/5	4Δ and 4Δ & 7Δ
5. ER AX4/6	5Δ, 5Δ & 2Δ, and 5Δ & 2-3Δ
6. ER AX5/7	6Δ
7. ER AX6/8	7Δ, 7Δ & 4Δ, 7Δ & 3-4Δ, 7Δ & 3-5Δ

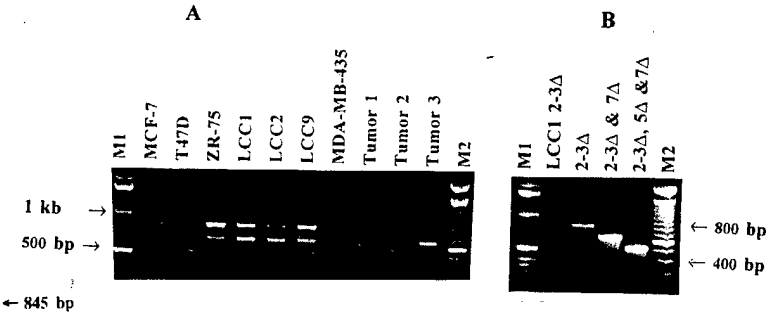
ER 2Δ SPLICE VARIANTS



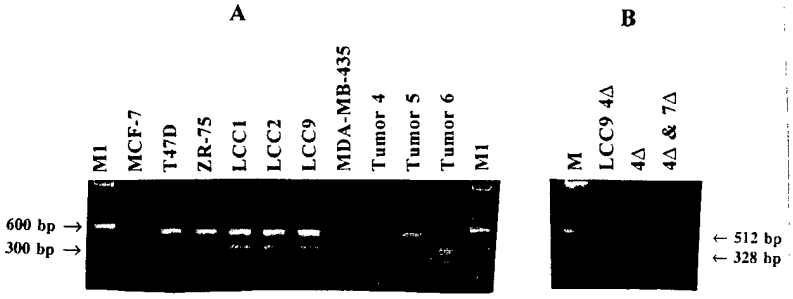
ER 3Δ SPLICE VARIANTS



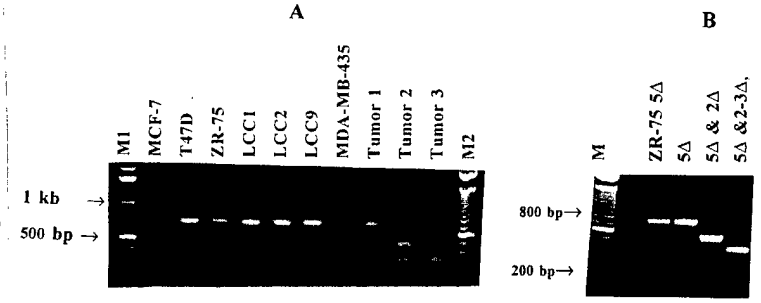
ER 2-3Δ SPLICE VARIANTS



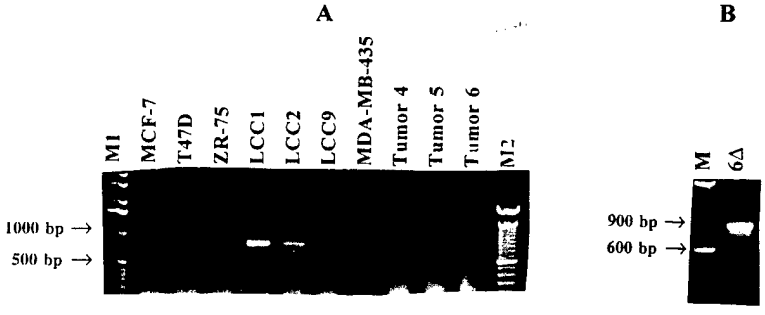
ER 4Δ SPLICE VARIANTS



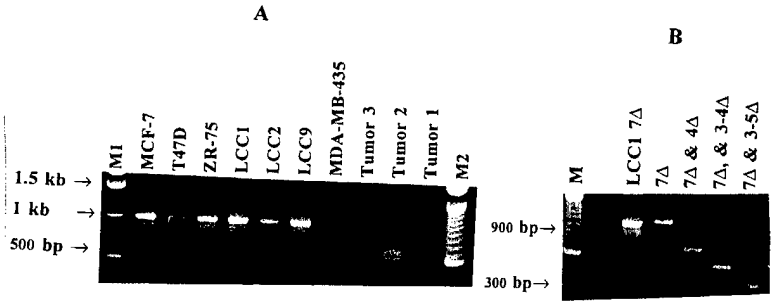
ER 5Δ SPLICE VARIANTS



ER 6Δ SPLICE VARIANTS



ER 7Δ SPLICE VARIANTS



Primer Design Strategies for the Targeted Amplification of Alternatively Spliced Molecules

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Received February 9, 1999

The alternatively spliced mRNAs are conventionally detected by RT–PCR approaches, which by virtue of specific primer design, are focused on small regions of the known wild-type mRNA. However, there are major problems in this approach, such as: (1) threshold of detection—a competitive amplification occurs among the wild-type and all the alternatively spliced molecules; therefore, the detection of a particular spliced molecule depends on the relative expression levels of its mRNA species within a sample; and (2) the mRNAs which have multiple deletions in distant exons such as an estrogen receptor (ER)[2] transcript which has deletions in exons 2 and 7 cannot be distinguished from transcripts having single deletions in exon 2 or 7 by this method (1).

To circumvent these problems, Yang and Le (2) described specific amplification of alternatively spliced molecules using targeted primers which have three of three unique overhang bases across the splice junction. However, in many instances there is often a complete or partial homology between the bases in the wild-type junctions and the alternate splice junctions. In these cases, it is necessary to target sequences beyond the first three bases of the splice junction to identify the unique bases. In the current study, we wanted to determine the number of extreme 3′ bases in the primer that were required to be unique and the number of bases the primer could extend beyond the junction and still maintain specific amplification of the target sequences. We designed and tested a series of primers using ER exons 7Δ, 5Δ, 2Δ, and 2–3Δ as models with plasmids containing their respective nucleotide sequences and full-length wild-type ER cDNA, pIC-ER-F. The construction of ER plasmids containing the exon 7-, 5-, 2-, and 2–3Δ sequences was accomplished as follows. First, their respective nucleotide sequences were coamplified with wild-type sequences using the cDNA prepared from the cell line MCF-7 (3) and cloned into pCRII-TOPO vector. The exon 7Δ sequence (688 bp) was amplified using a sense primer, ER1S, 5′-GCCCGCTCATGATCAAACGC-3′ (position: exon 4, bp 1113–1132), and an antisense primer, ER1A, 5′-TACTTTTGCAAGGAATGCGA-3′ (position: exon 8, bp 1977–1958), and cloned to obtain pCRII-TOPO-7Δ. The sequence and locations of all the primers described here are based on the ER cDNA sequence published by Green and others (4). The exon 5Δ nucleotide sequence (300 bp) was amplified using a sense primer, ER2S, 5′-GGAGACATGAGAGCTGCCAAC-3′ (position: exon 4, bp 1082–1102), and an antisense primer, ER2A, 5′-CCAGCAGCATGTCGAAGATC-3′ (position: exon 6, bp 1520–1501), and cloned to obtain pCRII-TOPO-5Δ. The exon 2Δ (468 bp) and exons 2–3Δ (350 bp) sequences were generated using the sense primer, ER3S, 5′-TGCCCTACTACCTGGAGAACG-3′ (position, exon 1, bp 615–635), and an antisense primer, ER3A, 5′-GGTCAGTAAGCCCATCATCG-3′ (position: exon 4, bp 1273–1254). The resulting exon 2Δ and exon 2–3Δ plasmids were termed as pCRII-TOPO-2Δ and pCRII-TOPO-2–3Δ, respectively. The PCRs were carried out using 50 ng of the test plasmid at initial denaturation conditions of 5 min at 95 °C followed by 94 °C for 1 min, annealing for 1 min at either 61 or 68 °C as indicated in the text, and extension for 2 min at 72 °C for 30 cycles plus a final extension for 10 min at 72 °C.

A series of antisense primers (as illustrated in Table 1), ER AX6/8-1 (positions: exon 8, bp 1804–1786, and exon 6, bp 1601–1600) through ER AX6/8-7 (positions: exon 8, bp 1798–1786, and exon 6, bp 1601–1594) which have varying number of overhanging bases at the 6/8 splice junction (Table 1A) were tested together with the sense primer, ER1S, at an annealing temperature of 61 °C. If the targeted primers specifically recognize only the 6/8 splice junction, we would expect them to generate a PCR product ranging from 508 bp for ER AX6/8-1 to 502 bp for ER AX6/8-7 with pCRII-TOPO-7Δ and no product with pIC-ER-F. On the other hand, if the exon 8 portion of the primers anneals and amplifies the wild-type sequences in pIC-ER-F, we would expect to observe a PCR product from 692 bp with ER AX6/8-1 to 686 bp with ER AX6/8-7. Similarly, if the exon 6 portions of the primers recognize and amplify exon 6 of the wild-type sequences in pIC-ER-F, we would expect to observe PCR products as described for the pCRII-TOPO-7Δ. The results are shown in Fig. 1A. Lanes 1–7 contain the PCR products amplified with the targeted primers ER AX6/8-1 through ER AX6/8-7, respectively, using pCRII-TOPO-7Δ as template and lanes 8–14 contain corresponding products with pIC-ER-F as the template. As seen in lanes

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[2] Abbreviations used: ER, estrogen receptor; exon Δ, exon deletion; AX, antisense; SX, sense.

Analytical Biochemistry **271,** 194–197 (1999)
Article ID abio.1999.4154
0003-2697/99 $30.00

TABLE 1

Design of Antisense Primers for Targeting ER Splice Variants, ER 7Δ and ER 5Δ

		Number of 3′ overhang bases		Target specific (at annel. temp)	
	Exon 8 Exon 6	Total	Unique in the extreme 3′ end	61°C	68°C
	A. Primers for exon 7Δ				
ER AX6/8-1	5′ ATGCTCCATGCCTTTGTTA// CA 3′	2	1/1	No[a]	N/T
ER AX6/8-2	5′ TGCTCCATGCCTTTGTTA// CAG 3′	3	2/2	No[a]	N/T
ER AX6/8-3	5′ GCTCCATGCCTTTGTTA// CAGA 3′	4	2/3	No[a]	N/T
ER AX6/8-4	5′ CTCCATGCCTTTGTTA// CAGAA 3′	5	3/4	Yes	N/T
ER AX6/8-5	5′ TCCATGCCTTTGTTA// CAGAAT 3′	6	4/5	Yes	N/T
ER AX6/8-6	5′ CCATGCCTTTGTTA// CAGAATT 3′	7	4/6	Yes	N/T
ER AX6/8-7	5′ CATGCCTTTGTTA// CAGAATTA 3′	8	5/7	Yes	N/T
Wild type	5 TGCCTTTGTTA// CTCATGTG 3′				
	Exon 8 Exon 7				
	B. Primers for Exon 5Δ				
	Exon 6 Exon 4				
ER AX4/6-1	5′ CACATTTTCCCTGGTTC// CTGG 3′	4	1/1	No[a]	—[c]
ER AX4/6-2	5′ ACATTTTCCCTGGTTC// CTGGC 3′	5	1/2	No[a]	Yes
ER AX4/6-3	5′ CATTTTCCCTGGTTC// CTGGCA 3′	6	2/3	No[a,b]	Yes
ER AX4/6-4	5′ ATTTTCCCTGGTTC// CTGGCAC 3′	7	3/4	No[b]	Yes
ER AX4/6-5	5′ TTTTCCCTGGTTC// CTGGCACC 3′	8	4/5	No[b]	Yes
ER AX4/6-6	5′ TTTCCCTGGTTC// CTGGCACCC 3′	9	5/6	No[b]	No[b]
ER AX4/6-7	5′ TTCCCTGGTTC// CTGGCACCCT 3′	10	6/7	No[b]	No[b]
Wild type	5′ CCCTGGTTC// CTGTCCAAGA..3′				
	Exon 6 Exon 5				

Note. N/T, not tested.

[a] Amplifies the wild-type exon at the 5′ end of the primer.

[b] Amplifies the wild-type exon at the 3′ end of the primer.

[c] Not applicable.

1–7, all the targeted primers generated the expected ~500-bp product with pCRII-TOPO-7Δ. However, when the template was pIC-ER-F, the targeted primers ER AX6/8-1 through ER AX6/8-3, which have fewer than three bases unique to the splice junction in the extreme 3′ end, generated an approximately 690 bp PCR product (lanes 8–10), indicating the annealing of these primers to exon 8 of the wild-type ER sequences. On the other hand, the primers ER AX6/8-4 through ER AX6/8-7, all of which have a minimum of three or more bases unique to the splice junction in the extreme 3′ end, did not generate any PCR products with pIC-ER-F (lanes 11–14). These results indicated the absence of amplification by their annealing to either exon 7/8 or 6/7 junctions in the wild type. Based on these results, it can be hypothesized that the ER 7Δ targeted primers which have a minimum of three of four unique bases at the extreme 3′ end will specifically amplify the spliced junction without amplifying the flanking exons. In addition, it appears that as many as eight bases past the exon 6/8 splice junction are permitted without amplification of the wild-type sequences.

Next, we tested the above hypothesis using antisense primers, ER AX4/6-1 (positions: exon 6, bp 1484–1468, and exon 4, bp 1328–1325) through ER AX4/6-7 (positions: exon 6, bp 1478–1468, and exon 4, bp 1328-1319) (Table 1B) for the specific amplification of ER exon 5Δ. As seen in Table 1B, both exon 4/6 and 5/6 junctions have the same initial three bases, CTG. Therefore, we designed primers further beyond the splice junction to target the unique sequences and tested together with the sense primer, ER1S, at an annealing temperature of 61°C. If the targeted primers are specific for the 4/6 junction, we would expect to observe PCR products ranging from 233 bp with ER AX4/6-1 to 227 bp with ER AX4/6-7 when amplifying pCRII-TOPO-5Δ and no product with pIC-ER-F. Alternatively, if the targeted primers anneal and amplify exon 6 or 4 of the wild-type junctions in pIC-ER-F, we would expect to observe PCR products of sizes approximately 370 or 230 bp, respectively. The PCR results with these primers are shown in Fig. 1B, a. Lanes 1–7 contain the PCR products amplified with the targeted primers ER AX4/6-1 through ER AX4/6-7, respectively, with pCRII-TOPO-5Δ and lanes 8–14 contain corresponding products with pIC-ER-F. As seen in lanes 1–7, all of the targeted primers generated the expected ~230-bp product with pCRII-TOPO-5Δ. However,

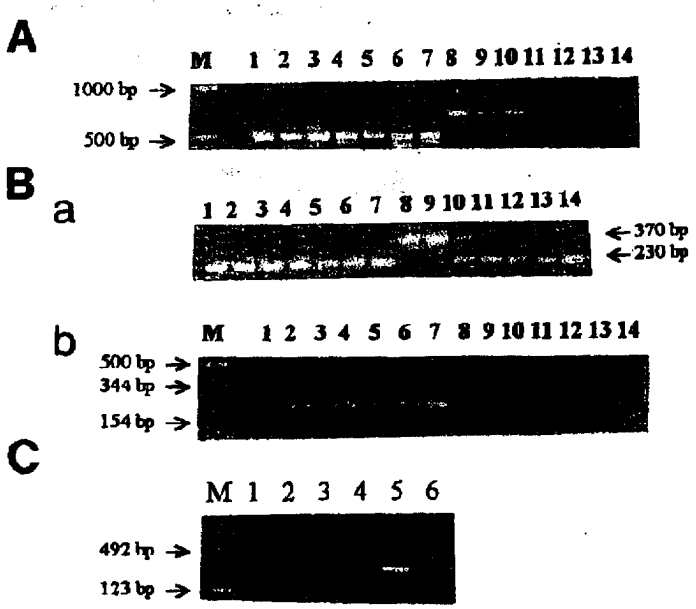

FIG. 1. Testing of targeted primers for the ER exon 6/8, 4/6, 1/3, and 1/4 splice junctions with plasmid templates containing their respective sequences and wild-type sequences. (A) Lanes 1–7 contain the PCR products amplified with the primers ER AX6/8-1 through ER AX6/8-7, respectively, using pCRII-TOPO-7Δ as the template and lanes 8–14 contain corresponding products using pIC-ER-F as the template. (B, a and b) a and b show the PCR products obtained at the annealing temperatures of 61 and 68°C, respectively, with the primers ER AX4/6-1 through ER AX4/6-7. In each of these panels, lanes 1–7 contain the PCR products amplified using pCRII-TOPO-5Δ as the template and lanes 8–14 contain the corresponding products using pIC-ER-F as the template. (C) Lanes 1–3 contain the PCR products amplified with ER SX1/3 and ER3A and using no DNA, pCRII-TOPO-2Δ and pIC-ER-F, respectively, as the templates. Lanes 4–6 contain the PCR products amplified with the primer, ER SX1/4, and ER 3A using no DNA, pCRII-TOPO-2-3Δ, and pIC-ER-F, respectively, as templates. In each of these panels, the lane M contains molecular weight standards.

when the template was pIC-ER-F, primers AX4/6-1 through AX4/6-3 generated an ≈370-bp PCR product, indicating the annealing of 5′ end of these primers to exon 6 and amplification of the wild-type ER sequences (lanes 8–10). The targeted primers, AX4/6-4 through AX4/6-7, generated an ~230-bp band with the wild-type plasmid, indicating that the 3′ end portion of these primers recognized and amplified exon 4 of the wild-type sequences (lanes 11–14). The primer ER AX4/6-3 generated both 370- and 231-bp products indicating that it amplified from both exons 4- and 6 of the wild-type sequences (lane 10). Since none of the targeted primers were completely specific at the annealing temperature of 61°C, we subsequently tested them at an annealing temperature of 68°C. The results are shown in Fig. 1B, b. The primers, ER AX4/6-2 through ER AX4/6-7 maintained their ability to amplify the targeted sequencesat 68°C (lanes 2–7) with pCRII-TOPO-5Δ. The PCR results with pIC-ER-F are shown in lanes 8–14. The primer ER AX4/6-1 did not recognize either pCRII-TOPO-5Δ or the pIC-ER-F (lanes 1 and 8, respectively). The primers, ER AX4/6-2 through ER AX4/6-5 did not generate any PCR products with pIC-ER-F (lanes 9–12), indicating their specificity to 4/6 junction at an annealing temperature of 68°C. The primers AX4/6-6 and AX4/6-7 amplified a minor product of size ~230 bp (lanes 13 and 14), indicating amplification of sequences in exon 4 of the wild type. Thus it appears that in addition to the number of unique overhang bases in the extreme 3′ end of the primer, the annealing temperatures also influence the specific amplification of the targeted sequences. The above-described results with targeted primers for ER exon 5 splice variant provide evidence for our earlier hypothesis that a minimum of three of four bases unique to the splice junction in the extreme 3′ end of the primer are required to specifically amplify the alternate splice junction. It also appears that in order to design such a primer, the overhang sequences can extend up to eight bases past the splice junction without annealing and amplification of the wild-type sequences.

Keeping these parameters in mind, we next designed the targeted primers, ER SX1/3, 5′-CGCCGGCATTCTACAG 1/3 GACAT-3′ (positions, exon 1, bp 669–684, and exon 3, bp 876–880) and ER SX1/4 5′-GCCGGCATTCTACAG 1/4 GGATAC-3′ (positions, exon 1, bp 670–684 and exon 4, bp 993–998), for the exons 1/3 and 1/4 splice junctions, respectively. Each of these primers satisfy the requirements of our target primer design based on the results described above. We next individually tested these two sense primers using the

antisense primer, ER3A, at an annealing temperature of 61°C. The results are shown in Fig. 1C. Lanes 1–3 contain the PCR products generated with the targeted primer ER SX1/3 and the antisense primer ER3A using no DNA, pCRII-TOPO-2Δ, and pIC-ER-F as templates, respectively. Similarly, lanes 4–6 contain the PCR products with the targeted primer, ER SX1/4, and the ER3A using no DNA, pCRII-TOPO-2-3Δ and pIC-ER-F respectively as templates. As seen in Fig. 1C, the targeted primers designed for the exon 2Δ and 2–3Δ molecules amplified only the targeted splice junctions and generated the expected 414 bp and 296 bp products (lanes 2 and 5, respectively) but did not generate any products with the ER wild-type sequences (lanes 3 and 6, respectively). Thus the targeted primers which meet the requirement of at least three of four unique bases in the extreme 3′ end exclusively amplify the alternate sequences. We believe that the principles developed in the current study with ER will have broad applicability to splice variants of a diverse range of genes.

Acknowledgments. This work was supported by grants from the National Cancer Institute (CA 71150), the Susan G. Komen Breast Cancer Foundation, and the Department of Defense (DAMD 17-94-J-4495) awarded to I.P.

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Quantitative Protein Precipitation from Guanidine Hydrochloride-Containing Solutions by Sodium Deoxycholate/Trichloroacetic Acid

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Received February 22, 1999

Guanidine hydrochloride (GdnHCl)[1] is often used in protein purification procedures for the solubilization of proteins aggregated in inclusion bodies or in studies on protein unfolding and refolding. The determination of protein concentrations in solutions containing GdnHCl by spectroscopic methods such as Lowry (1) or Bradford (2), however, mostly fails because of the influence of GdnHCl on the spectroscopic behavior of the chromophores. Even the protein determination by densitometric evaluation of Coomassie-stained SDS–PAGE gels is not possible since SDS and GdnHCl form aggregates. Heating or dilution of the samples prior to SDS–PAGE application may be helpful but the reliability of these approaches is doubtful. Possible methods for removal of GdnHCl are RP-HPLC or gel filtration which, however, are time consuming and result in a considerable dilution of the protein. Precipitation of the protein by trichloroacetic acid (TCA) and resolubilization may be an alternative but quantitative results are seldom obtained because of the strong tendency of GdnHCl to prevent aggregation/precipitation. We examined the sodium deoxycholate (NaDOC)/TCA precipitation procedure by Bensadoun and Weinstein (3) for GdnHCl-containing solutions and present here the benefit as well as the constraints for a quantitative recovery of protein samples. The degree of protein recovery was quantified by densitometric evaluation of Coomassie-stained SDS–PAGE gels.

Materials and Methods

Ribonuclease A (RNase A) was obtained from Sigma and purified to homogeneity on a Mono S FPLC column from Pharmacia. NaDOC was purchased from Sigma, GdnHCl was from ICN, and TCA was from Merck. All other reagents were the purest ones commercially available.

Precipitation. The samples were mixed with 1% aqueous NaDOC (1:10, by volume). Then 50% aqueous TCA (1:5, by volume) was added. After incubation at room temperature for 15 min the samples were centrifuged at 21,000*g* for 15 min. The supernatant was decanted and 100 μl acetone was added to the pellet followed by thorough vortexing. After incubation at −20°C for 15 min the samples were centrifuged at 21,000*g* for 15 min. The supernatant was decanted and the pellet was dried under nitrogen.

Resolubilization. The precipitates were incubated in 100 μl 50 mM Tris–HCl buffer, pH 8.0, containing 0–0.5 M GdnHCl at 25°C for 30 min with or without vortexing. For application to electrophoresis the precipitation procedure was repeated.

Electrophoresis and densitometric evaluation. Electrophoresis was carried out according to Schägger and von Jagow (4) but with 10, 14, and 18% acrylamide for the sampling, spacer, and separating gel. The protein pellets were dissolved in 15 μl sample buffer. Six or 9 μl, respectively, was applied to SDS–PAGE and gels were stained with silver nitrate or with Coomassie brillant

[1] Abbreviations used: GdnHCl, guanidine hydrochloride; TCA, trichloroacetic acid; NaDOC, sodium deoxycholate; RNase A, ribonuclease A.

Analytical Biochemistry **271,** 197–199 (1999)
Article ID abio.1999.4149
0003-2697/99 $30.00

Journal of Cancer Research and Clinical Oncology

Founded in 1903 as Zeitschrift für Krebsforschung

Springer International

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ALTERATIONS IN THE ESTROGEN RECEPTOR mRNA IN THE BREAST TUMORS OF AFRICAN AMERICAN WOMEN

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Key words: Estrogen Receptor, Exon truncations, Breast tumors and African American women

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Running Title: Estrogen receptor transcripts in the breast tumors of African American women

ABSTRACT

Several recent reports have shown that the mortality rate with breast cancer is about three times as high in African American women as other populations. In addition, the available data also indicate that the tumors are very aggressive and poorly differentiated with a very low frequency of hormone receptors. To gain an insight into the factors which may be responsible for their aggressive tumors, we investigated the transcript profiles of the estrogen receptor (ER), the most important prognostic factor in breast cancer, in the tumors derived from African American women. We analyzed twenty five immunohistochemically ER^+ and six ER^- malignant tumors for ER mRNAs by RT PCR using a number of primer pairs. For comparative purposes, twenty ER^+ malignant tumor tissues derived from Caucasian patients were also included. Our results showed that only 15 of the ER^+ tumors from African American women patients had full length wild type receptor transcripts and the others exhibited alterations/truncations in exon 8. We also found that the majority of tumors which had alterations/truncations in exon 8 did not express the naturally occurring, more abundant exon 7 deletion transcript. Most of the tumors expressed exon-2, exons 2-3, and exon 5 deletion variant transcripts. Unexpectedly, two of the six immunohistochemically ER^- tumors showed full length wild type receptor mRNAs but none of the variant transcripts.

INTRODUCTION

Recent reports indicate that the experience with breast cancer varies in different populations. The incidence of breast cancer is reported to be slightly lower in African American women compared to other women (1). However, the incidence seems to differ in different age groups. It is higher in young but lower in middle aged and older African American women compared to women of similar age groups in other populations (2). Trends in survival rate also seem to differ both by age and race. It was reported that the death rate from breast cancer for African American women under the age 65 has increased, while for women in a similar age group in other populations it has declined. Death rates have increased for all women over the age 65 and the increase is reported to be three times as high in African American women as others. Several reports indicate that this high mortality rate is not due to differences in socioeconomic status, stage of diagnosis, known risk factors or methods of treatment (3). It has been hypothesized that breast tumors across racial and ethnic groups could have different biologic characteristics which may account for survival disparities (4). However, such factors are not identified thus far. There also appears to be differences in tumor biology in different racial groups. Histochemical studies of the tumors derived from African American women have shown a higher incidence of poorly differentiated tumors and an increased frequency of nuclear atypia, higher mitotic activity, and tumor necrosis (5). Immunohistochemical studies have also shown that the frequency of estrogen- and progesterone receptor expression is significantly lower in the tumors of African American women (6). The presence of these receptors is generally associated with well or moderately

differentiated tumors and considered a good prognosis for longer survival and lower risk of tumor recurrence. The biological factors responsible for the poorly differentiated and aggressive tumors with lower frequency of hormone receptors which may contribute to lower survival in African American women are not known. In the current study, we examined the tumors derived from African American women for alterations in ER structure which may in part contribute to the aggressive nature of these tumors. We investigated the ER mRNA by reverse transcription and polymerase chain reaction (RT PCR) using a number of primer pairs. Our results described here show that only fifteen of the twenty five tumors which were diagnosed as ER^+ by immunohistochemistry had detectable full length wild type ER mRNAs and others showed alterations/ truncations in exon 8. Another observation is that most of the tumors which had exon 8 alterations/truncations did not express the naturally occurring, more abundant exon 7Δ variant mRNA.

MATERIALS AND METHODS

AmpliTaq PCR core kits and QIAquick gel extraction kits were from QIAGEN Inc., Santa Clara, CA. Reverse transcriptase kits were from Applied Biosystems, Foster City, CA. [α -^{32}P]dCTP (Specific Activity 3000Ci/mMole, Cat # AA0005) was from Amersham, Piscataway, NJ. The primers for amplifying ER and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) were synthesized at Gibco-BRL Life Technologies, Rockville, MD. Trizol reagent for total RNA isolation was purchased from Gibco-BRL Life Technologies. Diethyl Pyrocarbonate (DEPC) treated water was from Research Genetics, Huntsville, AL.

PCR quality water and Tris-EDTA buffer were from Biofluids, Rockville, MD. pCR®II-TOPO, a cloning vector for PCR amplified products was obtained from Invitrogen, Carlsbad, CA.

Breast tumor samples. The breast tumor samples from African American women patients were collected from Howard University Hospital and Providence Hospital in Washington D.C. Tumor collection protocols were approved by the respective IRB committees. The tumor samples were collected immediately after the surgery and frozen in liquid nitrogen. The ER status of the tumors collected from Howard University hospital and Providence hospital was obtained from the Tumor Registries of the respective hospitals. It was determined immunohistochemically by Oncotech laboratories using a monoclonal antibody against the NH_2-terminal domain (A/B region) of the receptor. A total of twenty-five ER^+ and six ER^- tumors derived from African American patients and twenty Caucasian patient tumor samples (obtained from the San Antonio Breast Tumor bank) were analyzed in the current study.

RNA isolation. Total RNA from African American patient tumors was isolated using Trizol reagent and the manufacturer's protocol. Briefly, the tumors were first ground to a powder using a cold, sterile pestle and mortar in the presence of liquid nitrogen. The pulverized tumor powder (50-100 mg) was suspended in 1 ml of Trizol reagent and homogenized with a hand held homogenizer and incubated at room temperature for 5 minutes to permit the dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added

to the above, shaken vigorously and centrifuged at 4°C for 15 minutes at 12,000 X g to collect the supernatant. Total RNA was precipitated from the supernatant by adding 0.5 ml of isopropanol. The precipitate was washed twice with 75% ethanol, dried briefly and dissolved in DEPC treated water. The RNA samples from ER^+ breast tumors of Caucasian patients were similarly isolated. The total RNAs from the ER^+ MCF-7 cell line and three ER^- negative cell lines, LCC6, MDA-MB-235 and MDA-MB-435 were also isolated using Trizol reagent as described above with a minor change. The cells were suspended by pipetting up and down in Trizol reagent instead of homogenization. This method yielded about 30 μg of total RNA per 100 mg of tumor tissue and 0.8-1.0 mg per 10^7 tissue culture cells. The integrity of the isolated RNAs was verified by electrophoresis in 1.5% agarose gels in Tris-Acetate EDTA buffer and the concentration was determined by measuring the optical density at 260 nm. Only those RNA samples which demonstrated intact RNA bands as visualized by ethidium bromide staining were chosen for the current study. The integrity of the RNA was further confirmed by RT PCR amplification of GAPDH transcripts.

Reverse Transcription and PCR. The isolated RNA was reverse transcribed to cDNA using Maloney Murine Leukemia Virus reverse transcriptase and random hexamers. Briefly, the standard reaction mixture contained 1 μg of total RNA, 2.5 units of reverse transcriptase, 1 mM each of dNTPs, 2.5 μM random hexamers, 1 U of RNAse inhibitor, 5 mM $MgCl_2$ and 1 X PCR buffer in a total volume of 20 μl. To reverse transcribe the RNA, the reaction tubes were first left at room temperature for 10 minutes, followed by incubations at 42^0 C for 15 minutes, 99^0 C for 5 minutes and finally 5^0 C for 5 minutes. For ER cDNA

amplification, various primer sequences located in exons 1, 4, 6, 7 and 8 as listed in the text were used. The positions of the primers are also shown schematically in Figure 1. The sequence and locations of all the primers described in the present study are based on the full length ER cDNA sequence published by Green and others (7). The Polymerase Chain Reactions were performed in an automatic thermal cycler (MJ Research) as described previously (8) in a 12.5 µl reaction volume containing the cDNA reverse transcribed from 125 ng of total RNA, 1 X PCR buffer, 1 X Q solution (Qiagen), 200 µM each of dNTPs, 2 µM each of sense and anti-sense primers and 0.6 U of Taq polymerase. The PCR conditions were initial denaturation for 1 minutes at 95^0 C followed by 94^0 C for 1 minutes, annealing for 1 minutes at 55^0 C, and extension for 2 minutes at 72^0 C for 40 cycles and final extension for 10 min at 72^0 C. The cDNA prepared from tumor samples were verified for the presence of GAPDH RNA using a sense primer,
5' AAGGCTGAGAACGGGAAGCTTGTCATCAAT 3' (position, exon 3, bp 241-270) and an anti-sense primer, 5'TTCCCGTCTAGCTCAGGGATGACCTTGCCC 3' (position, exon 7, bp 740 -711) (9) under the same PCR conditions as for ER. All of the primer pairs were first tested on the cDNA prepared from MCF-7 RNA and included as a positive control in all of the PCR amplifications. The RNA samples isolated from ER^- cell lines were used as negative controls.

Detection and sequence analysis of PCR products. To detect the PCR products, an aliquot of the reaction mixture (6 µl) was electrophoresed on 1% agarose gels and stained with ethidium bromide. To detect the variant ER products, the PCRs were conducted in the

presence of [α -^{32}P]dCTP at 0.5% of the total reaction volume and the radiolabelled PCR products were electrophoresed in a Bio-Rad vertical slab gel apparatus in 4.5% Polyacrylamide gels, 90 mM Tris-Borate and 0.2 mM EDTA at a 40 mA constant current for 3.5 hrs. The gels were dried in a Savant gel dryer and subjected to autoradiography using Kodak X-Omat AR film and DuPont NEN Lightning Plus intensifying screens at room temperature. In order to confirm the identity of the PCR amplified ER products, they were electrophoresed in agarose gels and purified using QIAquick gel extraction kit. The purified products were cloned into pCR®II-TOPO vector and sequenced by the cycle sequencing method (10, 11) on an automated DNA sequencer (carried out at Biopolymer Laboratory, University of Maryland School of Medicine, Baltimore, MD).

RESULTS

The ER mRNA profiles were studied by RT PCR in tumors which were diagnosed for ER status by immunohistochemistry. We analyzed twenty five ER^+ and six ER^- malignant tumors from African American women and twenty ER^+ malignant tumors from Caucasian patient tumors. To detect ER transcripts, we employed primer pairs which could amplify the exons 1-6, 1-4, and 4-8. When primers ER1S (5' CTCCACACCAAAGCATCTGGG 3', position, exon 1, bp 245-265) and ER1A (5' CCAGCAGCATGTCGAAGATC 3', position, exon 6, bp 1520-1501) which amplify exons 1-6 were used, twenty- three ER^+ and two ER^- tumors from African American patients, and all twenty Caucasian patient tumors gave the expected size PCR product (~ 1.275 kb) corresponding to the wild type ER. A PCR product

profile from representative tumor samples and MCF-7 is shown in Figure 2A. We did not detect any products in two of the ER^+ African American patient tumors either by ethidium bromide staining on agarose gels or as radiolabelled products on acrylamide gels. We analyzed exons 4-8 in all of the tumors using the primer pair ER2S (5' GCCCGCTCATGATCAAACGC 3', position, exon 4, bp 1113-1132) and ER2A (5' ATACTTTTGCAAGGAATGCGA 3', position, exon 8, bp 1978-1958). These primers amplified two products of sizes 866 bp and 682 bp in fifteen ER^+ African American patient tumors, and all twenty Caucasian patient tumors. The 866 bp and 682 bp products were identified as the wild type and exon 7 deletion variant by sequence analysis. Two of the ER^- African American patient tumors which were positive with primers ER1S and ER1A also amplified the 866 bp product but not the 682 bp product. A PCR product profile generated from MCF-7, a representative of 20 Caucasian patient tumors, a representative of fifteen ER^+ African patient tumors which gave positive products (Type I), a representative of eight ER^+ African American patient tumors which did not give any products (Type II) and a representative of two ER^- African American patient tumors are shown in Figure 2B. These results were repeatable in at least five different cDNA preparations from the tumor RNAs and several PCR trials. Two ER^+ tumors which did not give any products with ER1S and ER1A also did not yield any products with ER2S and ER2A either by ethidium bromide staining on agarose gels or as radiolabelled products on acrylamide gels. The reasons for the absence of any transcripts in these two tumors are not known, however, it should be noted that they showed very low levels (~ 20 f M) of protein by immunohistochemistry. These two tumors are probably false ER^+ and were not analyzed further. Thus it appears that only fifteen of the

twenty-five ER^+ African American patient tumors showed full length wild type sequences. Detection of full length wild type sequences in only fifteen of the twenty-five African American women patient tumors which were diagnosed as ER^+ by immunohistochemistry was unexpected. We hypothesized that the eight ER^+ Type II tumors which did not amplify with ER2S and ER2A may have alterations and/truncations in exon 8. Therefore, we next examined these tumors for potential alterations and/ or truncations in exon 8 with another antisense primer, ER3A (5' GCACTTCATGCTGTACAGATGC 3' position, exon 8, bp 1822-1801) in exon 8 upstream of ER2A together with the sense primer ER2S. The results are shown in Figure 3. The PCR products generated from MCF-7, a Caucasian patient tumor and a Type I African American patient tumor are also included as positive controls. Two of the eight Type II African American patient tumors gave a product which is slightly higher than the expected 710 bp wild type band but did not amplify the exon 7 deletion product (Type IIA). Two of the Type II tumors gave the expected wild type and exon 7 deletion products of sizes 710 bp and 526 bp respectively (Type II). One tumor did not amplify any product (Type IIC) and three tumors amplified only the expected wild-type ER band but not the exon 7 deletion product (Type IID). A PCR product profile from a representative of each of Type IIA-, B-, C-, and -D is shown in Figure 3. The gel is overloaded to show the absence of the naturally occurring exon 7Δ variant in Type IID tumors.

To test the possibility that type IIC tumor may have the whole exon 8 truncation, we designed another antisense primer, ER4A (5' GTCCTTCTCTTCCAGAGAC 3', position, exon 7, bp 1651-1633) in exon 7 and tested this together with ER2S. The results are shown in

Figure 4. The PCR products from MCF-7, a Caucasian patient tumor and Type I African American patient tumor are included as positive controls. The Type IIC tumor amplified an expected 538 bp wild type product and an additional 400 bp product which was identified as exon 5Δ. These results indicated the deletion of all of exon 8 in this tumor.

All of the above eight Type II African American patient tumors which did not amplify the expected PCR products with ER2S and ER2A were further examined for potential alterations using a sense primer in exon 7, ER3S, 5' CAGGCCTGACCCTGCAGCAGC 3' (position, bp 1710-1730) and an anti-sense primer in the non-coding region, ER5A, 5' TCTCCAAGTCACCAATTAAAGG 3' (position, bp 2474-2454). These two primers amplified an expected 764 bp product from MCF-7 and all Type I African American patient tumors but did not yield a corresponding band in any of the eight Type II tumors (data not shown).

Since the primer pair ER2S and ER4A co-amplified exon 5Δ along with wild type sequences, they were used to screen all of the tumors for the presence of 5Δ. We observed the presence of 5Δ in all of the Caucasian patient tumors, and twenty three ER^+ African American patient tumors. However, it is absent in two of the ER negative tumors which showed the presence of full length sequences. A representative of the ER^- negative tumor is shown in Figure 4. A minor band was visualized which is larger than the exon 5 deletion product but was not characterized because of its very low abundance.

To detect the exon deletion variants in the exon 1-4 region, we designed another set of primers, a sense primer, ER4S, (5' TGCCCTACTACCTGGAGAACG 3', position, exon 1, bp 615-635) downstream of ER1S and an anti-sense primer, ER6A, (5' GGTCAGTAAGCCCATCATCG 3', position, exon 4, bp 1272-1254) in exon 4, and screened all of the tumors for the presence of variant transcripts. The primers ER4S and ER6A amplified three PCR products of sizes 658 bp, 487 bp and 370 bp which were identified as the wild type, exon 2Δ and exon 2-3Δ transcripts respectively, by sequence analysis. The results from representative tumors are presented in Figure 5. All of the Caucasian patient tumors and nineteen ER^+ African American patient tumors showed the presence of both exon 2Δ and exons 2-3Δ transcripts. An additional three ER^+ African American patient tumors were positive for the exons 2-3Δ variant only. We did not observe any transcripts in any of the three ER^- cell lines tested with any of the primer pairs. Table 1 summarizes the expression of various transcripts in Caucasian and African American patient tumors. Two of the ER^- tumors which showed the presence of the full-length wild-type sequence did not show the presence of either 2Δ or exons 2-3Δ. A PCR product from one of the two ER^- tumors is shown in Figure 5. Thus it appears that the ER^- tumors which showed the presence of full length wild type PCR products did not express either of exon 5Δ, exon 7Δ, exon 2Δ or exons 2-3Δ variants.

DISCUSSION

We investigated the profiles of ER mRNA by RT PCR in the tumors of African

American women which are in general described as poorly differentiated and aggressive. We thought that the variations in the ER structure may, in part, contribute to the above characteristics. The results presented in this report clearly show that a considerable number of tumors contained mRNAs which appear to have alterations/truncations in the major portion of exon 8. One of the twenty five ER^+ tumors had a complete deletion of exon 8. These are the first observations showing alterations/truncations in ER exon 8.

Several studies have reported the presence of ER mRNAs which have truncations in exon 2, exon 3 and 6 in single breast tumors. The majority of truncated transcripts contain entire exon sequences of at least 2 of the 5' ER exon sequences, and then diverge into ER-unrelated sequences (12). The role of one of the truncated variants, namely clone 4 which consists of exons 1 and 2 of the wild type ER mRNA followed by unrelated sequences has been evaluated. It was found that the clone 4 level was significantly elevated in breast tumors compared to normal mammary gland (13). The relative level of this truncated variant was also found to be significantly elevated in breast tumors with characteristics of poor prognosis and endocrine resistance (14). These reports suggest that the truncated transcripts play a role in developing hormone resistance and influence the disease outcome.

The nature of truncations we have observed in the Breast tumors of African American women appear to be different from the above reported truncations in that the mRNAs appear to have intact wild type exons 1-7 but only a part of exon 8 is present. Since we could not obtain any product with primers which amplify between exon 7 and part of the non-coding

region in any of these tumors, it is possible that the non-coding sequence is also modified. Due to the limited amount of tumor RNA, further investigation by Northern Blots could not be undertaken to characterize this modification. Since the exon-8 sequences encode for a portion of domain E- and the complete F domain which controls ligand binding-, ligand-dependent dimerization, ligand-dependent transactivating and transcriptional responses to estrogens and anti-estrogens, alterations/truncations in this exon, therefore, will considerably alter the ligand binding properties of the receptor. Another distinguishing characteristic observed in the African American patient tumors is that the majority of tumors which showed the alterations/truncations in exon 8, did not express the naturally occurring, more abundant exon 7Δ. Although, *in vitro* studies have shown that exon 7Δ has a negative dominant effect on the wild type receptor (15), it was postulated that the variants, including the exon 7Δ, which are expressed in normal tissues play a role in the estrogen induced signal transduction pathway. The significance of the absence of the exon 7Δ is not known, but it may be relevant that this absence was observed only in tumors which did not demonstrate full length transcripts. Our results also show that two of the truncated tumors which did not express the more abundant exon 7Δ variant had lower mobility than the expected wild type product on polyacrylamide gels. This lower mobility may be due to base insertions. Due to a very limited amount of RNA from those tumors, the PCR products could not be cloned and sequenced to identify the site and nature of the modifications. However, since they generated the expected size PCR products with primers which amplify exons 1-6, it is possible that the modifications could be either in exon 7 or the untruncated portion of exon 8.

The presence of exon deletion variants between exons 1-6 was also investigated using primers which amplify exons 1-4 and exons 4-7. Our study indicates that the majority of tumors co-expressed exon-2Δ, exons-2-3Δ, and exon-5Δ transcripts. The occurrence of the exons 2-3Δ in both normal and cancer tissues has been reported by Leygue and others (15, 16). In a study of 100 tumors, they reported the presence of this multiple deletion variant in less than 10% of the samples. In our study we found that all but two tumors expressed this variant. The exon 2Δ variant was detected in 9 out of 15 tumors with primers ER3S and ER5A. The exon 5Δ variant transcripts were detected in twenty-three ER^+ African American patient tumors. These results show the co-expression of several exon deletion transcripts in the examined tumors.

Finally, very interesting observations were made with respect to immunohistochemically ER^- tumors. We found that two of six tumors studied showed full length ER mRNA sequences. We did not observe any products from ER^- cell lines, MDA-MB-435, MDA-MB-235 or LCC6 with any of the primer pairs tested. Unexpectedly, the above tumors did not express either exon 7Δ-, exon 2Δ-, exons 2-3Δ- or exon 5Δ transcripts. The physiological significance of these observations is not known. The presence of wild type full length sequences in these tumors suggests two possibilities: i) that the ER mRNAs are not translated in these tissues or ii) the protein amounts are in undetectable levels for immunohistochemistry. It is not known whether the absence of immunohistochemically detectable protein in these tumors is related to the absence of variant transcript molecules. It is possible that the lower frequency of ER^+ tumors reported in African American patients

may be due to the presence of altered receptors which are not translated to immunoreactive ER protein.

ABBREVIATIONS

ER, Estrogen receptor; GAPDH, Glyceraldehyde-3 phosphate dehydrogenase; DEPC, Diethyl Pyrocarbonate; RT PCR, Reverse transcription polymerase chain reaction; and Exon Δ, Exon deletion.

ACKNOWLEDGEMENTS

This work was supported by grants from the Susan G. Komen breast cancer foundation and the Department of Defence (DAMD 17-94-J-4495) awarded to I.P. The authors are thankful to Drs. Robert Dewitty, Lasalle D. Leffall, E. B Chung, and Robert E. Taylor at Howard university hospital for their help in collection of fresh tumors and Dr. Donna Williams for critical reading of the manuscript. Mrs. Arubala Reddy and Ms. Tamica Cooper are acknowledged for their assistance in tumor collection.

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FIGURE LEGENDS.

Figure. 1. Schematic representation of primers used in the current study for the amplification of various portions of estrogen receptor cDNA. The approximate primer locations in various exons are indicated with arrows.

Figure. 2. Amplification of ER exons 1-6 and 4-8 in African American and Caucasian patient tumors. The ER exons were amplified by RT PCR and the products were detected by electrophoresed in 1% agarose gels and ethidium bromide staining.

Panel A contains the PCR products generated with primers ER1S and ER1A, which amplify exons 1-6 from MCF-7, a representative of twenty Caucasian patient tumors, a representative of twenty three ER^+ African American patient tumors and a representative of two ER^- African American patient tumors. **Panel B** contains the wild type and exon 7 deletion variant PCR products generated with ER2S and ER2A which amplify exons 4-8 from MCF-7, a representative of twenty Caucasian patient tumors, a representative of fifteen ER^+ African American patient tumors which gave positive products (Type I), a representative of eight ER^+ African American patient tumors which did not give positive products (Type II) and a representative of two ER^- African American patient tumors.

Figure 3. Testing of ER^+ Type II African American patient tumors for possible truncations/alterations in exon 8. All the tumors which did not give any PCR products with ER2S and ER2A were tested using ER2S and an anti-sense primer, ER3A, upstream of ER2A

under the PCR conditions described in methods. The PCRs were conducted in the presence of [α -^{32}P]dCTP and the products detected by autoradiography. The PCR products from MCF-7, a representative from twenty Caucasian patient tumors, and a representative from fifteen Type I African American patient tumors are shown as positive controls. A representative from Type IIA, Type II, Type IIC and Type IID tumor products are shown.

Figure. 4. Testing of Type IIC ER$^+$ tumor for a possible complete deletion of exon 8. One of the ER$^+$ African American patient tumor which was positive for exons 1-6 but did not amplify any product between exons 4-8 was tested with primers which amplify exon 4-7. The amplifications were conducted in the presence of [α -^{32}P]dCTP and the product(s) were analyzed by autoradiography as described. The PCR products from MCF-7, a representative Caucasian patient tumor and a representative Type I African American patient tumor are shown as positive controls. The PCR product(s) from a representative ER$^-$ African American patient tumor which was positive for the full length wild type sequence is also shown along with Type IIC tumor.

Figure 5. Amplification of exons 1-4 to identify exon deletion variants in this region. To analyze the ER splice variants between exons 1-4, all tumor cDNAs were subjected to PCR amplification in the presence of [α -^{32}P]dCTP with primers ER3S and ER5A and the products were identified by autoradiography. The PCR products from MCF-7, a representative Caucasian patient tumor, a representative ER$^+$ African American patient tumor and a representative ER$^-$ African American tumor are shown.

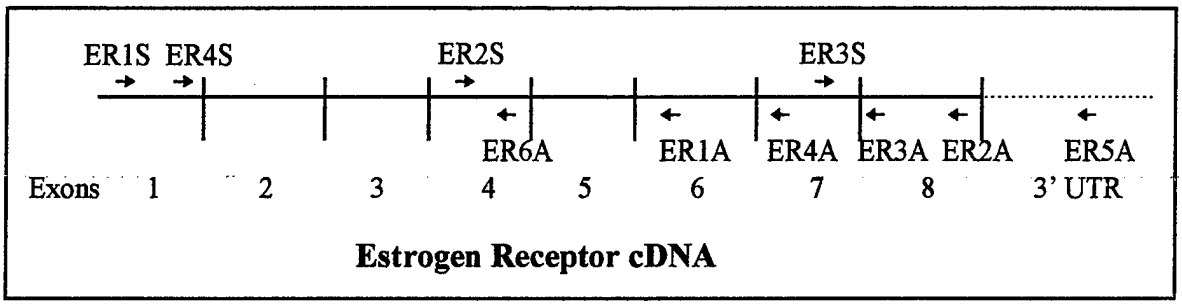
TABLE 1. ER TRANSCRIPT PROFILES IN CAUCASIAN AND AFRICAN AMERICAN BREAST TUMORS

#	Transcript	Number of Tumors Expressing the Transcript	
		Caucasian[1]	African American[2]
1.	Full length	20	15
2.	Exon 8 truncated	0	8
3	Exon 7Δ	20	17
4.	Exon 5Δ	2	23
5.	Exon 2Δ	20	19
6.	Exon 2-3Δ	20	22
7.	No transcript	0	2
8.	Complete exon 8 truncation	0	1

[1] Total number analyzed 20

[2] Total number analyzed 25

Figure. 1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ER2S
ER3S
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ER1A
ER4A
ER3A ER2A
ER5A
Exons 1 2 3 4 5 6 7 8 3' UTR
Estrogen Receptor cDNA

Figure 2

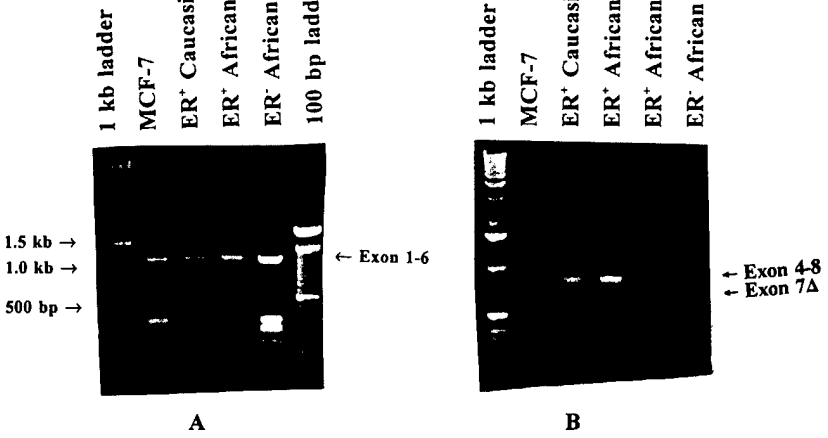
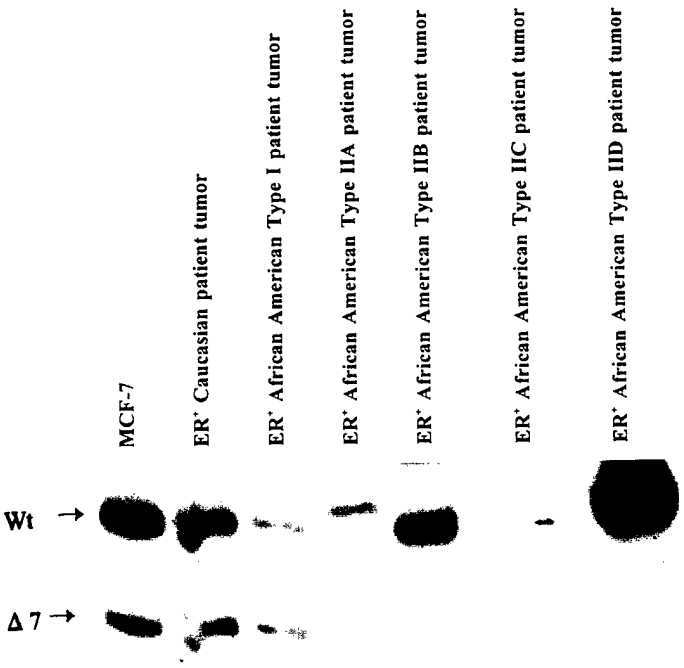


Figure 3



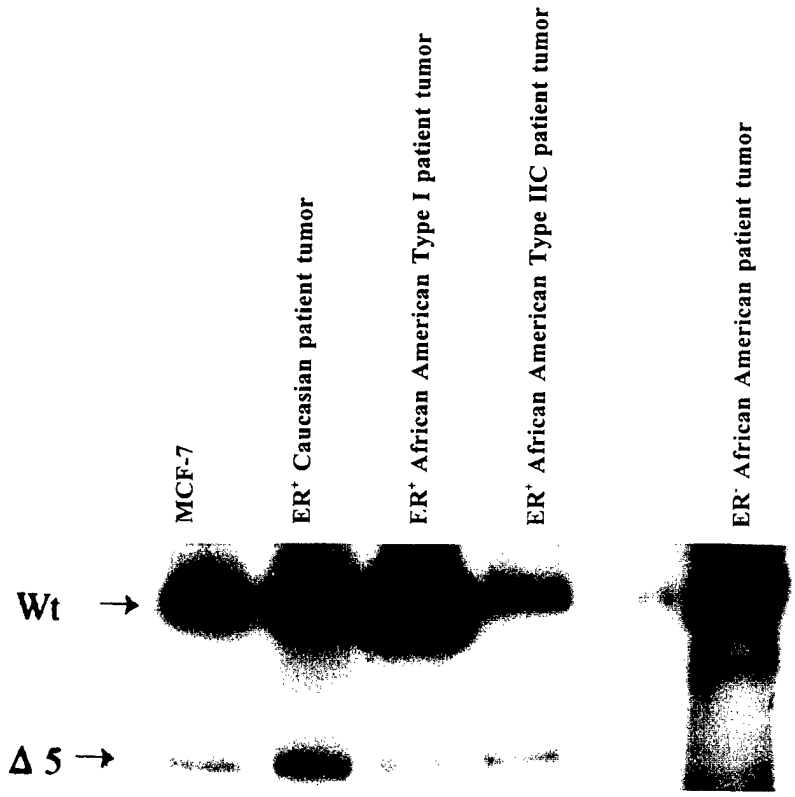


Figure 4

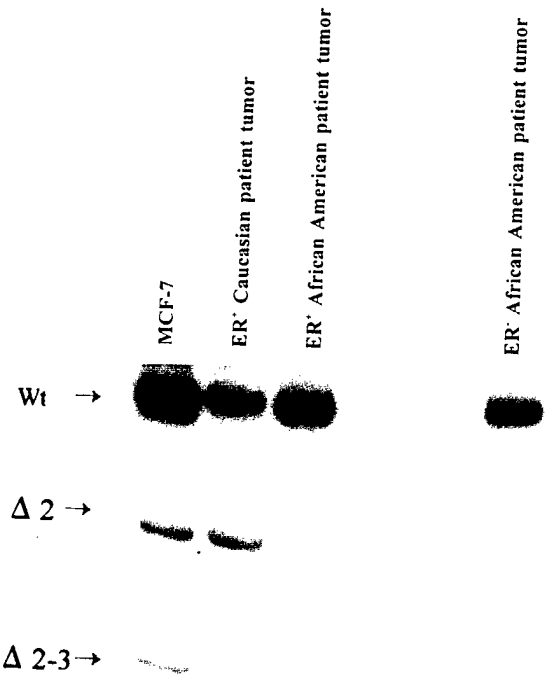


Figure 5